Sperm Chromatin Structure Assay (SCSA®)

35

Donald P. Evenson

Basic Protocol Steps

Fresh or frozen semen/sperm thawed in a 37°C water bath and diluted to $1–2 \times 10^6$ sperm/ml with TNE buffer:
0.01 M tris buffer
0.15 M NaCl
1 mM EDTA
pH 7.4

200 µl sperm suspension + 400 µl of:
0.15 M NaCl
0.08 N HCl
0.1% Triton–X 100
pH 1.20

After 30 s add 1.20 ml of:
0.20 M Na_2HPO_4
1.0 mM EDTA
0.15 M NaCl
0.10 M citric acid
6.0 µg AO/ml staining buffer
pH 6.0
Measure by flow cytometry

D.P. Evenson (✉)
SCSA Diagnostics, PO Box 107, 219 Kasan Ave, Volga, SD 57071, USA

Emeritus, South Dakota State University, Brookings, SD, USA

Department of Obstetrics and Gynecology, Sanford Medical School, University of South Dakota, Sioux Falls, SD, USA
e-mail: don@scsatest.com

Materials

Acridine Orange: (AO) *chromatographically purified* (Cat. # 04539, Polysciences, Inc., Warrington, PA 18976)
Automated solution dispensers: Oxford adjustable, 0.20–0.80 ml automatic dispenser for the acid-detergent solution with glass amber bottle (CAT # 13 687 65, Fisher Scientific, 800-766-7000) and Oxford adjustable, 0.80–3.0 ml automatic dispenser for the AO staining solution glass amber bottle (CAT # 13 687 66, Fisher Scientific).
Pipetters: adjustable 0–10 µl, 10–100 µl, 100–1,000 µl and a nonadjustable 200 µl
Ice buckets (3) for samples and reagent bottles
Water bath (37°C)
Stopwatch

Staining Solutions and Buffers

For solutions, use double distilled water (dd-H_2O). For sterilization, use a 0.22-mm filter. Use only the purest grade reagents. All solutions and buffers are stored at 4°C.

Acridine Orange (AO) Stock Solution, 1.0 mg/ml

Dissolved *chromatographically purified AO* (Polysciences) in dd-H_2O at 1.0 mg/ml can be stored up to several months. *Our laboratory has used only AO obtained from Polysciences, and*

A. Zini and A. Agarwal (eds.), *Sperm Chromatin: Biological and Clinical Applications in Male Infertility and Assisted Reproduction*, DOI 10.1007/978-1-4419-6857-9_35,
© Springer Science+Business Media, LLC 2011

thus, we have full confidence in this source. DO NOT use a more crude preparation of AO; failure will result. AO is a toxic chemical and precautions should be taken when handling it. Tare a 15-ml, flat-bottom scintillation vial on a 5-place electronic balance, carefully remove and transfer 3–6 mg AO powder from the stock bottle with a microspatula into the vial. Add an exact equivalent number of milliliters of water. Wrap the capped vial in aluminum foil to protect from light.

Acid-Detergent Solution, pH 1.20
20.0 ml 2.0 N HCl (0.08 N)
4.39 g NaCl (0.15 M)
0.5 ml Triton X-100 (0.1%)
H_2O to 500 ml
pH to 1.20 with 5 N HCl
Store up to several months

Use purchased 2.0 N HCl (e.g., Sigma Cat # 251–2); do not dilute from a more concentrated HCl solution that is likely less pure and may be of questionable strength. The Triton-X stock solution is very viscous. We use a wide-mouth pipette and carefully draw up the exact amount, wipe the outside of the pipette free of Triton-X, and then expel with force in and out of the pipette until all is dispensed.

0.1 M citric acid buffer
21.01 g/L citric acid monohydrate (F.W. = 210.14; 0.10 M)
H_2O to 1.0 L
Store up to several months at 4 C.

0.2 M Na_2PO_4 buffer
28.4 g sodium phosphate dibasic (F.W. = 141.96; 0.2 M)
H_2O to 1.0 L
Store up to several months at 4 C.

Staining buffer, pH 6.0
370 ml 0.10 M citric acid buffer
630 ml 0.20 M Na_2PO_4 buffer
372 mg EDTA (disodium, FW = 372.24; 1 mM)
8.77 g NaCl (0.15 M)
Mix overnight on a stir plate to insure that the EDTA is entirely in solution.
pH to 6.0 with concentrated NaOH pellets
Store up to several months

Slowly and carefully adjust the pH using very small pieces (cut with a scalpel and handled with a forceps) of concentrated NaOH pellets. Note that when the 0.2 M Na_2PO_4 buffer is removed from the refrigerator, salt crystals will be present. Heat in 37°C water bath until the salts are fully dissolved.

AO staining solution
600 µl AO stock solution is added to each 100 ml of staining buffer. Rinse the pipette tip several times. This solution is kept in a glass amber bottle.
Store up to 2 weeks at 4°C.

AO equilibration buffer
400 µl acid-detergent solution
1.20 ml AO staining solution

This is run through the instrument for ≈15 min prior to sample measurement to insure that AO is equilibrated with the sample tubing. This is also run through the instrument between different samples to maintain the AO equilibrium and help clean the prior sample out of the lines.

TNE buffer, 10×, pH 7.4
9.48 g Tris-HCl (FW = 158; 0.01 M)
52.6 g NaCl (FW = 58.44; 0.15 M)
2.23 g EDTA (disodium, FW = 372.24; 1 mM)
pH to 7.4 with 2 N NaOH
Store up to 1 year at 4 C

TNE buffer, 1×, pH 7.4
60 ml 10× TNE
H_2O to 600 ml
Check pH (7.4)
Store for several months at 4 C

FCM Tubing Cleanser (for unclogging FCM sample lines)
50% ETOH
50% household bleach (contains ~5% sodium hypochlorite)
0.5 M NaCl
Store at room temperature

50% household bleach (for eliminating AO from sample lines)
50 ml household bleach (~5% sodium hypochlorite)
50 ml H_2O

Sheath fluid
2× H_2O 0.45 nm filtered water + 0.1% Triton X-100 (this helps minimize bubbles in the flow channel). It is NOT necessary to use commercially sold sheath fluid unless one FCM sorts the sperm in a jet-in-air sorter.

Major Equipment

Ultracold freezer (−70 to −110°C) or, preferably, a LN_2 tank
Biological safety hood

Flow Cytometer(s)

The flow cytometer must have 488 nm excitation wavelength and an approximate 15–35 mW laser power. Fluorescence of individual cells is collected through red (630–650 nm long pass) and green (515–530 nm band pass) filters.

Orthogonal flow cytometer configuration and related signal artifacts. The highly condensed mammalian sperm nucleus has a much higher index of refraction than sample sheath (water) in a flow cytometer. This differential, coupled with the typical nonspherical shape of sperm nuclei and their orientation in the flow channel, produces an optical artifact consisting of an asymmetric, bimodal emission of DNA dye fluorescence when measured in orthogonal configuration flow cytometers where the collection lenses are situated at right angles to both sample flow and excitation source. Since DFI is a computer calculated ratio of red to total (red + green) fluorescence, the optical artifact of AO-stained sperm measured in the orthogonal instruments does not significantly interfere with results, and the DFI frequency histogram is very narrow for a normal population of sperm. Although each type of flow cytometer with different configurations of lens and fluidics produces different cytogram patterns, the DFI data are essentially the same.

The variables of DFI are useful especially, as discussed above, for toxicology and has been shown for animal fertility studies. Future studies will show its importance for human fertility studies. However, a simple determination of the percent of cells with denatured DNA (%DFI) and the percentage of cells with abnormally high green stainability (%HDS) can be reasonably estimated without the ratio calculations. %DFI is currently the most used variable of this assay for human fertility assessment.

Cell Preparation

Collection and Handling

Human semen samples are typically obtained by masturbation into plastic clinical specimen jars preferably after ~2 days abstinence. Of importance is the length of the previous abstinence period; if days of time have elapsed, then sperm stored in the epididymis can become apoptotic in which case such a sample would not be representative of a fresh semen sample. We suggest that a patient ejaculate, wait for two days, and ejaculate again, then the sample for testing be taken after another two days, e.g., ejaculate on Monday and Wednesday and collect clinical sample on Friday. Freshly collected semen should be quick-frozen as soon as liquefaction has occurred in about a half hour. The majority of semen samples may be kept for up to several hours at room temperature prior to measuring/freezing without significant loss of quality, allowing for collections within a medical institution and transport to the flow cytometry unit. However, we have observed in limited studies that an estimated 10% of samples have an increased DNA fragmentation while setting at room temperature; likely, these samples have very low antioxidant capacity. If transport is required outside of a building complex, the sample may be conveyed in an insulated box or jacket pocket to keep from freezing or on liquid ice if the ambient temperature is hot. Once a sample has been diluted in TNE buffer it should be measured or frozen immediately.

Freezing

After allowing ~30 min for semen liquefaction at room temperature, aliquots of raw or TNE diluted

($1–2 \times 10^6$ sperm/ml) semen can be frozen directly without cryoprotectants in an ultracold freezer (−70 to −110°C; 0.5–1.5 ml snap-cap tubes), a shipping box with dry ice, or can be placed directly into a LN_2 tank (cryovials). Samples should be frozen in vials that are approximately ¼ larger in volume than the semen volume to reduce the air–surface interface, thus minimizing related reactive oxygen damage. Keep the tubes vertical when freezing, since samples frozen at the bottom of a tube could be later thawed in a water bath with greater ease and safety. Cryoprotectants are not needed, since quick-frozen cells and those frozen with a cryoprotectant provide equivalent SCSA data. This feature is unique to mammalian sperm cells due to the highly condensed, crystalline nature of the nucleus.

Flow Cytometer Setup

Workstation

The SCSA procedure requires that samples are thawed and processed in the immediate vicinity of the flow cytometer. The following equipment should be handy for quick and easy use.

- Ice buckets containing wet ice to hold the reagent bottles, sample tubes, and TNE buffer
- Disposable gloves
- Stopwatch
- Automatic pipetters and tips
- Reagent bottles deeply embedded in the ice buckets containing wet ice
- Container with disinfectant for sample disposal

Flow Cytometer Alignment

Prior to measuring experimental samples, the instrument must be checked for alignment using standard fluorescent beads. Very importantly, an AO equilibration buffer (400 µl acid-detergent solution and 1.20 ml AO staining solution) must be passed through the instrument sample lines for ≈15 min prior to establishing instrument settings. This insures that AO is equilibrated with the sample tubing. To save time, this AO buffer can be run through the instrument during its warm-up time prior to alignment and again just before measuring samples. Contrary to existing rumors, using AO in a flow cytometer *does not* ruin it for other purposes. The sample lines DO NOT need to be replaced after using AO in a flow cytometer! However, the system DOES need to be fully equilibrated with AO, as AO does transiently adhere to the sample tubing by electrostatic force, thus reducing the required AO concentration. After finishing SCSA measurements, AO can easily be cleansed from the lines by rinsing the system for about 10 min with a 50% filtered household bleach solution followed by 10 min of filtered H_2O. Our laboratory has utilized many fluorescent dyes and sample types after measuring AO stained sperm without any associated problems.

Reference Samples

Because SCSA variables are very sensitive to small changes in chromatin structure, studies on sperm using this protocol require very precise, repeat instrument settings for all comparative measurements whether done on the same or different days. These settings are obtained by using aliquots of a single semen sample called the “reference sample” (this is not a “control” sperm from a fertile donor). A semen sample that demonstrates heterogeneity of DNA integrity (e.g., 15% DFI) is chosen as a reference sample and then diluted with cold (4°C) TNE buffer to a working concentration of $1–2 \times 10^6$ cells/ml.

CLIA and other licensing agencies, e.g., New York Health, require that for every measurement period that a low %DFI and a high %DFI sample become part of the measurement data.

Several hundred 300-µl aliquots of this dilution are immediately and quickly placed into 0.5-ml snap-cap vials and flash frozen at −70 to −100°C in a freezer or, preferably in a LN_2 tank. These reference samples are used to set the red and green photomultiplier tube (PMT) voltage gains to yield the same mean red and green fluorescence levels from day to day. The mean red and green fluorescence values are set at ≈125/1,000 and

≈475/1,000 channels, respectively. The values established by a laboratory (preferably the same as above) should be used consistently thereafter. Strict adherence to keeping the reference values in this range must be maintained throughout the measurement period. A freshly thawed reference samples is measured after every 5–10 experimental samples to insure that instrument drift has not occurred.

Very few FCM protocols are as demanding as the SCSA for using a reference sample. Obviously, it would be advantageous to prepare a new batch of reference samples from the same individual donor. However, if a new donor is used, then first set the PMTs for the previous reference sample to be in the same position and then measure the new reference sample and note the red and green mean values and use these values for further studies.

Since reference samples can be stored in LN2 for years, a donor could provide enough samples for thousands of reference aliquots.

Sample Measurement

Single frozen samples are immersed in a 37°C water bath, just until the last remnant of ice disappears. When analyzing a series of human samples, it is extremely helpful to obtain the sperm count in advance of SCSA preparation so that time is not lost determining the proper dilution. However, if a sample(s) needs to be measured quickly for a clinical decision, then rather than wait for a sperm count, estimate a dilution, check the flow rate, and if necessary, resample with the proper dilution to attain the required flow rate of ~200 events per second. *A 200-ml aliquot of fresh or frozen/thawed semen sample of known sperm concentration* is placed into a 12 × 75 mm conical plastic test tube. Then, 400 μl of the acid-detergent, low pH buffer is added with an automatic dispenser setting deep in the ice bucket. This dispenser needs to be highly accurate and to have a maximum volume capacity only a small volume more than what is being dispensed. At the beginning of sample measurement and after long breaks in measurement, dispense several volumes from both dispensers before starting with the samples, as AO in the delivery tube may have been damaged by light and solutions in the plastic delivery tubes may be warmer than 4°C. A stopwatch is started immediately after the first buffer is dispensed. Exactly 30 s later, the AO staining solution is added. The sample tube is then placed into the flow cytometer sample chamber – which varies in design by different instruments. The sample flow is started immediately after placing it in the sample holder. Using the stopwatch that was started with the addition of the acid-detergent solution, the acquisition of list mode data to computer disk is started at 3 min. This allows ample time for AO equilibration in the sample and hydrodynamic stabilization of the sample within the fluidics, both very important aspects of AO staining. The sperm flow rate is checked during this time, and if it is too fast, i.e., >250 cells, a new sample is made at the appropriate dilution. This protocol provides approximately equal to two AO molecules/DNA phosphate group. Thus, to initially set up the proper hydrodynamic conditions, measure several sperm samples that have a predetermined cell count of ≈1.5×10^6 sperm/ml (or known concentration of fluorescent beads) and adjust the flow rate settings (if possible) for ≈200 cells/beads per second. On a FACScan, the "low flow" rate setting delivers an approximate correct flow rate. If a sample's flow rate is too high, this same sample cannot be diluted with AO buffer to lower the concentration. Sample and sheath flow valve settings of the instrument are never changed during these measurements so the liquid flow rate is constant. Doing so widens the flow sample stream with consequential loss of resolution. Thus, a change in sperm count rate is a function of sperm cell concentration only. PMT settings should be fairly identical from day to day depending on slight alignment differences between days and sample runs. *All samples are measured at least twice in succession* for statistical considerations and data on ~5,000 sperm cells (total events recorded are higher due to debris) are recorded per measurement. For the second measurement, take the sample from the same thawed aliquot; dilute appropriately, process for the SCSA and

measure. After the second measurement of a sample is finished, place a tube of AO equilibration buffer on the instrument to maintain the AO conditions and wash any of the previous sample out of the tubing and start preparing the next sample. There is no need to run this buffer between the duplicate measurements of the same sample; just allow the first one to stay running while preparing the second one.

Gating and Debris Exclusion

A very important*, but* sometimes difficult point, is deciding where to draw the computer gates to exclude cellular debris signals (signals located at the origin in the red (X) vs. green (Y) fluorescence cytograms) from the analysis. This gate is usually best set at a 45° angle, i.e., at the same channel value for both red and green fluorescence values. Resolution of debris and sperm signal is partly instrument dependent.

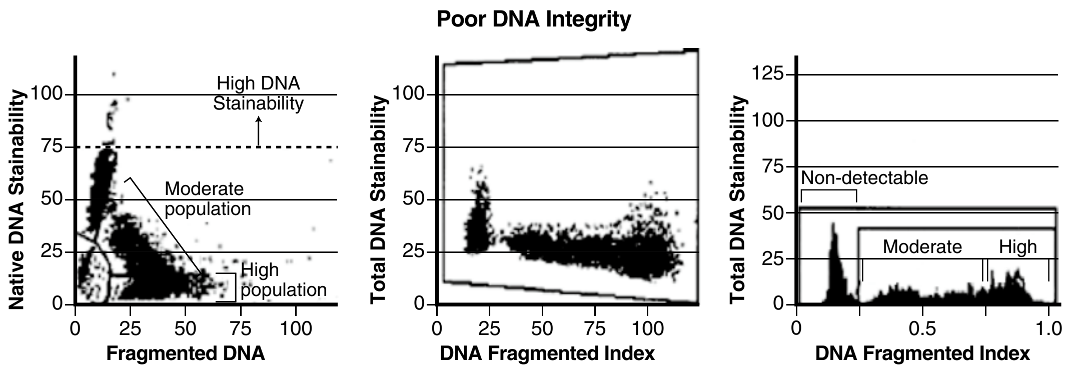
The real SCSA values of a sample cannot be learned if the fluorescence from debris (i.e., free cellular components and other contaminants) is blended in with the sperm fluorescence signal. This can sometimes be eliminated by washing the sperm or processing though gradients. However, there is a risk of losing cell types and the advantage of using whole semen measurements is then compromised. Bacterial debris appears as a straight line to the left of and parallel with the main sperm population in the cytograms; this usually can be gated out, but not in all samples.

Critical Parameters or Points

Computer gating to determine % DFI and % HDS

The left hand panel of the figure below shows how %DFI and %HDS can be calculated by placing computer gates to the right of the cigar-shaped pattern of sperm without DNA fragmentation (%DFI) as well as the % of sperm with increased green fluorescence (%HDS) characteristic of immature sperm and/or sperm with altered protein composition (Fig. 35.1).

As discussed in the SCSA chapter, it is easy to obtain the %DFI from a semen sample represented



Patient	Date	Measurement	DFI	SD DFI	% DFI	% HDS
7272-113	## ##	1	563.7	307	64.9	6.4
		2	561.4	304.8	64.9	7.2
		mean	562.6	605.9	64.9	6.8
		sd	1.2	1.1	0	0.4

Fig. 35.1 The *middle* and *right hand panels* show the effects of SCSAsoft® calculations without computer gating for %DFI, which is calculated from the DFI frequency histogram as shown in the *right-hand panel*

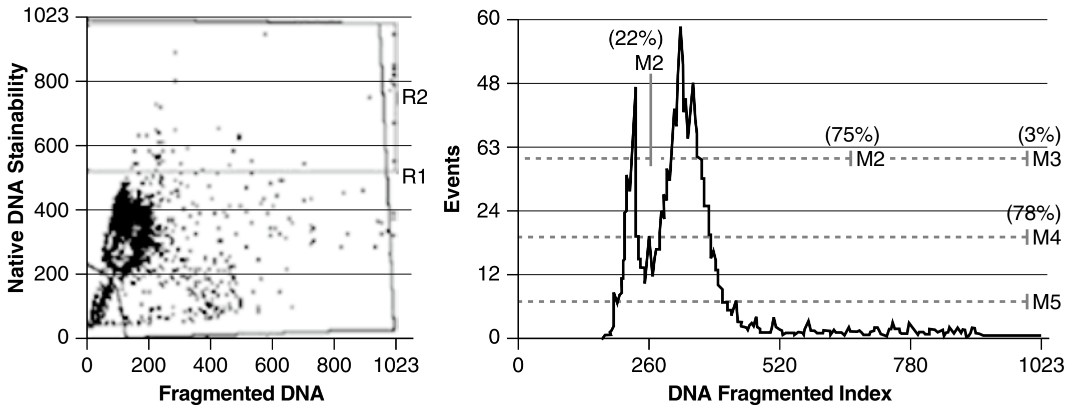


Fig. 35.2 SCSA® data from a sample with a high frequency of sperm with moderate DNA fragmentation. In this case, it is impossible to gate between sperm with no or moderate DNA fragmentation in the FCM dot-plot (*left panel*). With the SCSAsoft®, gating between the two populations is unproblematic (*right panel*, 34)

in the above panel. However, in the semen sample represented in the panel below (Fig. 35.2), it is more difficult to obtain the correct %DFI without the use of SCSAsoft®. The %HDS is equally calculated with or without SCSAsoft®.

In summary, the SCSA protocol appears rather simple offhand; however, there are numerous very critical points that, unless followed exactly, will give very poor data and serious errors in clinical diagnosis and prognosis.