V ANALYTICAL METHODS

P. Thyroxine (T4) in Serum
Aria HT

1. Principle

Radioimmunoassay is based on the competition between radiolabeled and unlabeled antigen (or hapten) for a specific antibody. Unlabeled antigen from standard or unknown samples competes with a fixed concentration of labeled antigen for a limited number of antibody binding sites. Increasing amounts of unlabeled antigen result in an increased amount of unbound labeled antigen. The bound radioactivity is therefore inversely related to the concentration of the antigen in the patient sample or standard.

In the Aria HT Thyroxine System, the equilibrium described above is attained through a conventional soluble incubation of diluted serum in the presence of radioactive labeled antigen and a soluble monoclonal antibody specific for thyroxine. This incubation mixture then flows through a solid-phase elutable adsorbent. The labeled and unlabeled thyroxine not bound to the soluble monoclonal antibody during incubation are bound to the solid-phase adsorbent, whereas the labeled and unlabeled thyroxine bound to the soluble monoclonal antibody during the incubation flows through the adsorbent and into the bound flow cell where the labeled thyroxine molecules are counted by a gamma detector. An eluting agent then passes over the adsorbent to free the bound thyroxine. The thyroxine (labeled and unlabeled), freed from the adsorbent by the eluting solution, flows into the free flow cell where it is counted by the gamma detector. Following this elution, the adsorbent is rinsed and made ready for reuse in the next cycle. Because this technique is automated, the separation conditions are highly uniform.

2. Specimen

a. Use only particulate-free serum. Sample assayed should not contain any visible clotted material.

b. Minimum sample size is 60 ul. Optimum size is 120 ul.

c. Make sure that clinical samples contain no countable radioactivity, since it may lead to erroneous results.

d. If specimens are not to be used immediately, cover and store at 2-8 ^{o}C. If specimens are not to be used within 24 hours, they should be frozen at -20 ^{o}C.

All sample containers should be capped when not in use.

Reconstituted controls should be stored at 2-8 °C, and they are stable for 7 days.

6. Procedure

a. All reagents and samples must be brought to room temperature before being used, but to minimize deterioriation, do not leave at this temperature longer than necessary.

b. The standard curve is run by using single standard level points. Participant and control samples may be run in duplicate or singly and should be run after a standard curve has been run.

c. Standard curve preparation: Volume of standard dispensed depends on the number of samples to be assayed. The Aria HT will restandardize as programmed by the operator. The chamber binding has been shown to be stable; ±3%, up to 100 cycles. Each restandardization is performed from the same sample cups:

Volume to Dispense	No. of Aspirations
At least: 60 ul	1
75 ul	2
100 ul	3, etc.

Overlay dispensed standards with 50 ul LiquiCap. To make sure that the LiquiCap is dispersed, tip the tray.

d. Preparation of controls.

One to three controls can be run on the Aria HT. The volume of control serum required is determined by the number of times each control is to be assayed, as entered by the operator in the test run parameters. Each control can be assayed several times from the same sample cup. Add the appropriate amount of each control to separate sample cups. Overlay the dispensed controls with 50 ul LiquiCap. To make sure that the LiquiCap is dispersed, tip the tray.

e. Preparation of participant samples

If samples have been frozen, thaw, mix and recentrifuge after thawing at 1000 X g for 5 minutes (2-25 °C). Dispense 0 standard in sufficient volume for the aspirations programmed, (120 ul for two warm-ups is recommended) into the first cup of the unknowns. This is the warm-up position. Add a minimum of 60 ul of each participant serum (120 ul for duplicate assays from the same cup) to subsequent sample cups. Overlay the dispensed samples with 50 ul LiquiCap. To make sure that the LiquiCap is dispersed, tip the tray.

f. For instrument set-up instructions, see Aria HT Instruction Manual.

7. Calculation

Using an iterative technique, the HT computer determines certain standard curve parameters which are used to determine concentrations in samples. The CHC computer will calculate the FTI (free thyroxine index), using the formula:

(T3U X T4)/100 = FTI

8. Reference

Becton Dickinson. Instruction manual for determination of thyroxine using the Aria HT. Orangeburg, New York: Becton Dickinson, 1986; RMNo. 71308-R1.

V. ANALYTICAL METHODS

Q. Dehydroepiandrosterone Sulfate (DHEA-S) in Serum

1. Principle

The Serono DHEA-S radioimmunoassay (RIA) procedure is based on the competitive binding principles of radioimmunoassay.

DHEA-S (unlabeled antigen) in serum samples, standards and controls competes with 125-I DHEA-S (labeled antigen) for a limited number of DHEA-S antibody binding sites.

At the end of the reaction period, centrifugation is used to separate bound DHEA-S from free DHEA-S, both labeled and unlabeled. After decanting, the antibody-bound DHEA-S remains in the tube as a precipitate and is counted in a gamma scintillation counter calibrated to detect 125-I. Participant results may then be quantitated by comparison with the standard curve.

DHEA-S is the major C-19 adrenal steroid secreted primarily by the zona reticularis of the adrenal cortex. Although this androgen is biologically weak, because of its high concentration in serum, it contributes significantly to androgenization. ACTH and, possibly other pituitary factors, regulate DHEA-S secretion.

2. Specimen

a. No preparation of participants is required. Specimens from non-fasting persons are acceptable.

b. 100 ul of serum is required. Plasma samples are not recommended for this assay.

c. Grossly hemolyzed or lipemic specimens are not acceptable.

d. If specimens are not to be used immediately, cover with Parafilm, and place in refrigerator. Serum specimens are stable for up to 24 hours at 2-8 °C. For longer storage (up to 90 days), store in aliquots at -20 °C. Avoid repeated freezing and thawing. Do not attempt to thaw specimens by heating in a water bath.

3. Reagents, Supplies, and Storage

All reagents, standards, and controls are stored in the RIA refrigerator at 2-8 °C. Refer to the expiration date on bottles. Test components are sufficient for 100 tests.

a. DHEA-S antiserum
color code: blue — 50 ml rabbit anti-DHEA-S serum in buffer, bovine serum albumin, 0.1% sodium azide, and inert dye.

b. DHEA-S 125-I tracer
color code: red — 100 ml containing <10 microcuries 125-I labeled DHEA-S in buffer, bovine serum albumin, polyethyleneglycol, goat antirabbit gamma-globulin, and 0.1% sodium azide.

c. DHEA-S standards — 0.3 ml containing 0, 50, 200, 1,000, 4,000, or 8,000 ng/ml DHEA-S in human serum with 0.1% sodium azide.

d. 12- x 75-mm disposable polypropylene test tubes.
e. Test-tube rack.
f. Accuprep pipetting station with a 100-ul and 1-ml syringe.
g. Vortex mixer.
h. Sorval RC-3 automatic refrigerated centrifuge.
i. Isodata gamma counter calibrated to detect 125-I.

4. Quality Control Material

Two levels of Lyphochek controls, level II and "Lyphodil," will be run in duplicate, one level each at the beginning and end of each analytical run.

To reconstitute the product, add 5.0 ml of type I water with a class A volumetric pipette to the desired vials. Replace the rubber stopper and allow the reconstituted material to stand at least 15 minutes. After that time, invert the bottle several times to ensure that the contents are thoroughly mixed. Before initial use, inspect each vial carefully for undissolved material. Reconstituted controls should be stored at 2-8 °C, and they are stable for 7 days.

Reconstitute the Lyphochek controls as above. To the Lyphochek level I add exactly 1.0 ml of Lyphochek level III, using a 1-ml class A volumetric pipet. Mix thoroughly and label as "Lyphodil Control."

5. Procedure

Action	Result
1. Allow all standards, controls, reagents, and samples to come to room temperature. Mix by gentle inversion. Place the 100-ul syringe on the Accuprep channel 1 position for sample pipetting.	

Action	Result
2. Label 12- x 75-mm assay tubes in duplicate for each participant sample, control, standard, nonspecific binding, and total count.	
3. Prime the 100-ul syringe in channel 1 35 times with water.	
4. Remove the Accuprep channel 2 line from water. Press PRIME CHANNEL, ENTER, RUN.	This will prime the line five times with air to remove water from the line.
5. Accuprep channel 1 line should be in water. Place channel 2 line in DHEA-S tracer.	
6. PRIME channel 2 line 2 times into waste and 20 times back into the reagent bottle.	First prime will remove any residual water from line.
7. Press PROGRAM, 11 ENTER.	This will bring up the DHE1 program.
8. Pipette the nonspecific binding tubes.	
a. Press hand control switch.	This will pull up 75 ul of water into channel 1 and 1,000 ul of tracer into channel 2.
b. Press hand control switch again.	10 ul of air will be drawn into channel 1 to separate the wash water from the sample.
c. Wipe tip and place probe tip in the 0 standard.	
d. Press hand control switch.	15 ul of sample will be drawn into channel 1.
e. Wipe tip and place probe tip into nonspecific binding tube.	
f. Press hand-control switch.	10 ul of sample and 1,000 ul of tracer will be dispensed into tube.
g. Place probe tip in waste container.	
h. Press hand-control switch.	Channel 1 will rinse itself with 75 ul of water and remove the extra 5 ul of sample.

Action	Result
i. Repeat steps 8 a-g for second NSB tube.	
9. Pipet the total count tubes.	
a. Press hand-control switch twice.	This will draw up water and air into channel 1 and tracer into channel 2.
b. Let probe tip remain in air.	
c. Press hand-control switch.	Instead of sample, only air will be drawn into channel 1.
d. Place probe tip in total count tube.	
e. Press hand-control switch.	Tracer only will be dispensed into the tube.
f. Place tip in waste container.	
g. Press hand-control switch.	To rinse the line.
h. Repeat steps 8 a-g for second total count tube.	
i. Cover these tubes with Parafilm and set aside.	
10. Pipette standards, controls, and participant sample tubes.	
a. Press hand-control switch	75 ul of water will be pulled into channel 1 and 100 ul tracer into channel 12.
b. Press hand-control switch again.	10 ul of air will be drawn into channel 1 to separate wash water from the sample.
c. Place probe tip in desired standard, control, or participant sample.	
d. Press hand-control switch.	15 ul of specimen will be drawn into channel 1.
e. Place probe tip into appropriately labeled tube.	
f. Press hand-control switch.	10 ul of specimen and 1,000 ul of tracer will be dispensed.

Action	Result
g. Place probe tip into waste.	
h. Press hand-control switch.	To rinse line.
i. Repeat step 9 for all standards, controls, and participant samples.	
j. Vortex all tubes.	
11. When all tubes are completed, EXIT the program.	
12. Replace the 100-ul syringe on channel 1 with a 1,000-ul syringe.	
13. Before removing the channel 1 line from water, press PROGRAM 12, ENTER.	This will bring up the DHE2 program.
14. Complete the nonspecific binding tubes.	
a) Press hand-control switch.	This will pull up 1,000 ul of water into channel 1. (Nonspecific binding tubes receive water instead of antiserum.)
b) Place probe tip in nonspecific binding tube.	
c) Press hand-control switch for each tube.	This will dispense 500 ul of water into each tube.
d) Place the nonspecific binding tubes aside.	
15. EXIT the program.	
16. Remove Accuprep channel 1 line from water and remove channel 2 line from tracer.	
17. Place channel 2 line into water and PRIME six to seven times into waste.	This will wash the tracer out of the line. More complete cleaning will be done later.
18. PRIME channel 1 line five times with air.	This will remove any residual water from line.
19. Place channel 1 line into DHEA-S antiserum. PRIME 2 times into waste and 20 times back into reagent bottle.	

Action	Result
20. Press PROGRAM, 12, ENTER.	This will bring up the DHE2 program.
21. Press hand-control switch.	1,000 ul of antiserum will be drawn into channel 1.
22. Place probe tip into all assay tubes in turn. Press hand-control switch for each tube.	500 ul of antiserum will be dispensed. When syringe is empty, it will automatically fill.
23. Repeat until all tubes are completed.	
24. EXIT the program.	
25. Vortex mix all tubes.	
26. Incubate tubes at room temperature for 30 minutes. They should be covered with Parafilm to prevent evaporation.	
27. While tubes are incubating, remove channel 1 line from antiserum. Place in water. PRIME 20 times into waste to clean.	
28. Place channel 2 line in TraceKlean. PRIME 20 times into waste. Place line in water and PRIME 20 times into waste.	This line is now clean.
29. After tubes have incubated, spin for 15-20 minutes at 3,000 rpm in Sorval RC-3 centrifuge at 20-25 ^{o}C.	
30. Decant tubes carefully. Allow to drain on paper towel for 2 minutes. Blot tubes carefully with a Kimwipe.	
31. Read in gamma counter. Refer to Isodata operational instruction for specifics.	

6. Reference

Diagnostic Systems Laboratories. DHEA-S RIA kit protocol. Webster, Texas: Diagnostic Systems Laboratories, 1984.

V. ANALYTICAL METHODS

R. Follicle-Stimulating Hormone (FSH) in Serum

1. Principle

The principle of the Leeco FSH assay is a classic double antibody radioimmunoassay system. Follicle-stimulating (FSH) from participant samples, standards, and controls is simultaneously reacted with a constant amount of antihuman follicle-stimulating hormone antisera and with 125-I-labeled FSH. The amount of tracer-labeled FSH binding is inversely proportional to the amount of FSH present in the participant specimen, standard, or control. The antibody-bound tracer is separated by a second antibody and precipitation accelerator. After centrifugation and decanting, the antibody bound tracer that remains in the tube as a precipitate is counted in a gamma scintillation counter.

2. Specimen

a. Participant preparation consists of determining whether the participant has undergone radiation therapy and, if so, whether the radiation will affect the assay.

b. 0.5 ml serum is necessary.

c. Grossly hemolyzed samples should not be used.

d. If specimens are not to be used immediately, cover with Parafilm and place in refrigerator. Serum aliquots are stable at 2-8 °C for 48 hours. If longer storage is required, store at -20 °C. Avoid repeated freezing and thawing.

Do not attempt to thaw specimens in a heated water bath.

3. Reagents, Supplies, and Storage

a. Store all reagents, standards and controls at 2-8 °C in the RIA refrigerator. Refer to the expiration date on the bottles.

b. FSH 125I tracer solution-- 20 ml of 125-I labeled follicle-stimulating hormone, less than 10 microcuries. Contains 0.075M phosphate buffer, pH 7.5, and 0.1% sodium azide.

c. FSH antiserum-- 20 ml of lyophilized >1:1,000 rabbit antihuman follicle-stimulating hormone (beta subunit specific). Contains 0.075 M phosphate buffer and 0.1% sodium azide.

d. FSH standards-- 2.0 ml each of 5, 10, 25, 50, 100, 200, mlU/ml of follicle-stimulating hormone in a bovine serum base and 0.1% sodium azide.

e. Precipitating antibody solution -- 50 ml containing precipitation accelerator and goat antirabbit antibody, 0.05% bovine serum albumin, and 0.1% sodium azide.

f. 12- X 75-mm polypropylene test tubes.
g. Test-tube rack.
h. Accuprep automatic pipet.
i. Vortex mixer.
j. 37 °C water bath.
k. Sorvial RC-3 centrifuge.
l. Gamma scintillation counter.

4. Quality Control Material

Two levels of Lyphochek controls, levels II and III, will be run in duplicate, one level each at the beginning and end of each analytical run.

To reconstitute the product, add 5.0 ml of type I water with a class A volumetric pipet to the desired vials. Replace the rubber stopper and allow the reconstituted material to stand at least 15 minutes. After that time, invert the bottle several times to ensure that the contents are thoroughly mixed. Before initial use, inspect each vial carefully for undissolved material. Reconstituted controls should be stored at 2-8 °C and are stable for 7 days.

5. Procedure

Action	Result
1. Allow all standards, controls, reagents, and participant samples to come to room temperature. Mix by gently inverting the containers.	
2. Label 12- X 75-mm assay tubes in duplicate for each standard, control, participant sample, nonspecific binding, and total count.	
3. Pipet the nonspecific binding tubes.	
a. Enter PROGRAM, 04.	

Action	Result
b) Press hand-control switch twice.	This will pull up water and air into channel 1 and water into channel 2. (nonspecific binding tubes do not receive antiserum).
c) Place probe tip into 0 standard.	
d) Press hand-control switch.	205 ul of 0 standard will be drawn into channel 1.
e) Place probe tip into nonspecific binding tube.	
f) Press hand-control switch.	200 ul of sample and 200 ul of water will be dispensed.
g) Place probe tip into waste.	
h) Press hand-control switch.	To rinse line.
i) Repeat steps 9 d-j for second nonspecific binding tube.	
4. EXIT the program.	
5. Remove the Accuprep channel 2 line from water. Press PRIME, CHANNEL, ENTER, RUN.	This will prime the line five times with air to remove water from line
6. Accuprep channel 1 line should be in water. Place channel 2 line in FSH antiserum.	
7. PRIME channel 2 line 2 times into waste and 20 times back into reagent bottle.	First prime will remove any residual water from line.
8. Press PROGRAM, 04, ENTER.	This will bring up the FSH1 program.
9. Pipet the standards, controls, and participant sample tubes.	
a) Press hand-control switch.	This will pull up 785 ul of water into channel 1 and 200 ul of antiserum into channel 2.
b) Press hand-control switch again.	10 ul of air will be drawn into channel 1. This will separate the wash water from the sample.
c) Place probe tip into the standard, control, or participant sample to be aspirated.	

Action	Result
d) Press hand-control switch.	205 ul of specimen will be drawn into channel 1.
e) Place probe tip into the appropriately labeled tube.	
f) Press hand-control switch.	200 ul of specimen and 200 ul of antiserum will be dispensed into each tube.
g) Place probe tip into waste.	
h) Press hand-control switch.	Channel 1 will rinse itself with 785 ml of water and remove the extra 5 ul of sample.
i) Repeat step 7 for all standards, controls, and participant samples.	
10. EXIT the program.	
11. Vortex mix the tubes and incubate at 37 ^{o}C for 30 minutes. Cover tubes with Parafilm to prevent evaporation.	
12. While tubes are incubating, remove channel 1 line from water and PRIME with air five times.	This will remove the water from the line.
13. Place line into FSH tracer. PRIME 2 times into waste and 20 times back into reagent bottle.	First prime will remove any residual water from line.
14. When incubation is complete, press PROGRAM, 05, ENTER.	This will bring up the FSH2 program.
15. Press hand-control switch.	1,000 ul of tracer will be drawn into channel 1.
16. Place probe tip into each tube in turn, including the total count tubes. (After dispensing tracer into the total count tubes, cover and set aside.)	
17. Press hand-control switch for each tube.	200 ul of tracer will be dispensed into each tube. When the syringe is empty, it will automatically refill.

Action	Result
18. Repeat until all tubes are completed. EXIT the program.	
19. Vortex mix all tubes and incubate at room temperature for 90 minutes. Cover tubes with Parafilm to prevent evaporation.	
20. While tubes are incubating, remove channel 1 line from tracer. Place line into Beckman TraceKlean and PRIME 20 times into waste. Then place line into water and PRIME 20 times into water.	To remove tracer from the line.
Note: The repeat Eppendorf pipet may also be used to add 0.5 ml of precipitating reagent. (See steps 21-28.)	
21. PRIME channel 1, five times with air.	To remove water from line.
22. Place channel 1 line into the FSH precipitating reagent. PRIME 2 times into waste and 20 times back into the reagent bottle.	
23. After tubes have incubated, press PROGRAM, 06, ENTER.	This will bring up the FSH3 program.
24. Press hand-control switch.	1,000 ul of reagent will be drawn into channel 1.
25. Place probe tip into each tube in turn.	
26. Press hand-control switch for each tube.	500 ul of reagent will be dispensed into each tube.
27. Repeat until all tubes are complete.	
28. EXIT the program.	
29. Vortex mix all tubes and spin for 20 minutes at 3,000 rpm at 2-8 °C in the Sorval RC-3 centrifuge.	
30. Decant tubes carefully. Do not allow to remain inverted for more than 1 minute.	

Action	Result
31. Count in the Isodata gamma counter, calibrated to detect I-125.	
32. Remove channel 1 line from precipitant reagent. Place in water and PRIME 20 times into waste to clean.	

6. References

Leeco Diagnostics. FSH-quant diagnostic kit test protocol. Southfield, Michigan: Leeco Diagnostics, 1984.

V. ANALYTICAL METHODS

S. Luteinizing Hormone (LH) in Serum

1. Principle

The principle of the Leeco LH-Quant is that of a classic double antibody radioimmunoassay (RIA) system. Luteinizing hormone (LH) from participant serum, standards, and controls is simultaneously reacted with a constant amount of antihuman LH antiserum and with 125-I-labeled LH. The amount of tracer-labeled LH binding is inversely proportional to the amount of LH in the sample. The antibody bound tracer is separated from the free tracer by a second antibody and precipitation accelerator. After contrifugation and decanting, the antibody-bound tracer, which remains in the tube as a precipitate, is counted in a gamma scintillation counter.

2. Specimen

a. Participant preparation consists of determining whether the participant has undergone radiation therapy and, if so, whether the radiation will affect the assay.

b. 0.5 ml serum needs no additives or preservatives.

c. Grossly hemolyzed samples should not be used.

d. If specimens are not to be used immediately, cover with Parafilm and place in refrigerator. Serum aliquots are stable at 2-8 °C for 48 hours. If longer storage is required, store at -20 °C. Avoid repeated freezing and thawing. Do not attempt to thaw specimens by heating in a water bath.

3. Reagents, Supplies, and Storage

a. Store all reagents, standards, and controls at 2-8 °C in the RIA refrigerator. Refer to the expiration date on the bottles.

b. LH 125-I tracer solution -- 20ml of 125-I-labeled LH (less than 10 microcuries). Contains 0.75M phosphate buffer, pH 7.5, and 0.1% sodium azide.

c. LH antiserum -- 20 ml of lyophilized >1:1,000 rabbit antihuman LH. Contains 0.075M phosphate buffer and 1% sodium azide. Reconstitute with 20 ml of distilled water.

d. LH standards -- 2.0 ml each of 5, 10, 25, 50, 100, and 200 mIU/ml and 5.0 ml of 0.0 mIU/ml of LH in a bovine serum base and 0.1% sodium azide.

e. Precipitation antibody solution -- 50 ml containing precipitation accelerator and goat antirabbit antibody with 0.5% bovine serum albumin and 0.1% sodium azide.

g. 12- X 75-mm polypropylene test tubes.
h. Test tube rack.
h. Accuprep antomatic pipette.
i. Vortex mixer
j. 37 °C water bath
k. Sorval RC-3 centrifuge.
l. Gamma scintillation counter.

4. Quality Control Material

Two levels of Lyphochek controls, levels I and II, will be run in duplicate, one level each at the beginning and end of each analytical run.

To reconstitute control material, add 5.0 ml of type I water with a class A volumetric pipette to the desired vials. Replace the rubber stopper and allow the reconstituted material to stand at least 15 minutes. After that time, invert the bottle several times to ensure that the contents are thoroughly mixed. Before initial use, inspect each vial carefully for undissolved material. Reconstituted controls should be stored at 2-8 °C and are stable for 7 days.

5. Procedure

Action	Result
1. Allow all standards, controls, reagents, and participant samples to come to room temperature. Mix by gentle inversion.	
2. Label 12- X 75-mm assay tubes in duplicate for each standard, control, participant sample, nonspecific binding, and total count.	
3. Prepare to pipet the nonspecific binding tubes.	
a. Enter PROGRAM, 08, and press run. (Pipet nonspecific binding tubes.)	

Action	Result
b. Press hand-control switch.	This will pull up water and air into channel 1 and water into channel 2 (nonspecific binding tubes do not receive antiserum).
c. Place probe tip into 0 standard.	
d. Press hand-control switch.	205 ul of 0 standard will be drawn into channel 1.
e. Place probe tip into nonspecific binding tube.	
f. Press hand-control switch.	200 ul of sample and 200 ul of water will be dispensed.
g. Place probe tip into waste.	
h. Press hand-control switch.	To rinse line.
i. Repeat steps 9 d-i of the FSH procedure for second nonspecific binding tube.	
4. EXIT the program.	
5. Remove the Accuprep channel 2 line from water. Press PRIME, CHANNEL, ENTER, RUN.	This will prime the line five times with air to remove water from line.
6. Accuprep channel 1 line should be in water. Place channel 2 line in LH antiserum.	
7. PRIME channel 2 line 2 times into waste and 20 times back into reagent bottle.	First prime will remove any residual water from line.
8. Press PROGRAM, 08, ENTER.	This will bring up the LH1 program.
9. Pipet the standards, controls, and participant sample tubes.	
a. Press the hand-control switch.	This will pull up 785 ul of water into channel 1 and 200 ul of antiserum into channel 2.
b. Press the hand-control switch again.	10 ul of air will be drawn into channel 1. This will separate the wash water from the sample.

Action	Result
c. Place probe tip into the standard, control, or participant sample to be aspirated.	
d. Press hand-control switch.	205 ul of specimen will be drawn into channel 1.
e. Place probe tip into the appropriately labeled tube.	
f. Press hand-control switch.	200 ul of specimen and 200 ul of antiserum will be dispensed into each tube.
g. Place probe tip into waste.	
h. Press hand-control switch.	Channel 1 will rinse itself with 785 ul of water and remove the extra 5 ul of sample.
i. Repeat step 7 for all standards, controls, and participant samples.	
10. EXIT the program.	
11. Vortex mix the tubes and incubate at 37 °C for 30 minutes. Cover tubes with Parafilm to prevent evaporation.	
12. While tubes are incubating, remove channel 1 line from water and PRIME with air five times.	This will remove the water from the line.
13. Place line into LH tracer and PRIME 2 times into waste and 20 times back into reagent bottle.	First prime will remove any residual water from line.
14. When tubes have completed the incubation, press PROGRAM, 09, ENTER.	This will bring up LH2 program.
15. Press hand-control switch.	1,000 ul of tracer will be drawn into channel 1.
16. Place probe tip into each tube, including the total count tubes. (After dispensing tracer into the total count tubes, cover and set aside.)	

Action	Result
17. Press hand-control switch for each tube.	200 ul of tracer will be dispensed into each tube. When the syringe is empty, it will automatically refill.
18. Repeat until all tubes are completed. EXIT the program.	
19. Vortex mix all tubes and incubate at room temperature for 90 minutes. Cover tubes with Parafilm and prime remaining tracer back into vial.	
20. While tubes are incubating, remove channel 1 line from tracer. Place line into Beckman TraceKlean and PRIME 20 times into waste. Then place line into water and PRIME again 20 times into waste.	To remove tracer from the line.
Note: The repeat Eppendorf pipet may also be used to dispense precipitating reagent. (See steps 21-28.)	
21. PRIME channel 1, five times with air.	To remove water from the line.
22. Place channel 1 line into the LH precipitating reagent. PRIME 1 time into waste and 20 times into reagent bottle.	
23. After tubes have incubated, press PROGRAM, 10, ENTER.	This will bring up the LH3 program.
24. Press hand-control switch.	1,000 ul of reagent will be drawn into channel 1.
25. Place probe tip into each tube in turn.	
26. Press hand-control switch for each tube.	500 ul of reagent will be dispensed into each tube.
27. Repeat until all tubes are complete.	
28. EXIT the program.	
29. Vortex all tubes and spin for 20 minutes at 3,000 rpm at 2-8 ^{o}C in the Sorval RC-3 centrifuge.	

Action	Result
30. Decant tubes carefully; do not allow to remain inverted for more than 1 minute.	
31. Count in the Isodata gamma counter, calibrated to detect 125-I.	
32. Remove channel 1 line from precipitant reagent. Place in water and PRIME 20 times into waste to clean.	

6. Reference

Leeco Diagnostics. LH-quant diagnostic kit test protocol. Southfield, Michigan: Leeco Diagnostics, 1984.

V. ANALYTICAL METHODS

T. Testosterone in Serum

1. Principle

The principle of the Leeco Testosterone method is that of a standard, double antibody radioimmunoassay (RIA). An aliquot of participant sample containing endogenous steroid is combined with a 125-I-labeled testosterone derivative. An aliquot of rabbit (antihuman) testosterone antibody is then added and incubated 1 hour. This solution also contains a lipolytic enzyme preparation to solubilize lipids present in the sample. The free testosterone is separated from bound testosterone (labeled and unlabeled) by the addition of goat antirabbit gamma globulin aided by polyethyleneglycol. Centrifugation and gamma scintillation measurement of the resultant precipitate complete the assay.

2. Specimen

a. No preparation of participants is required. Specimens from nonfasting persons are acceptable.

b. 500 ul of serum is required. Plasma collected with EDTA is acceptable.

c. Grossly hemolyzed specimens should not be used.

d. Samples from participants receiving radioisotopes for diagnostic or therapeutic procedures may also be unacceptable. All participants' sera should be screened for exogenous radioactivity.

e. Serum and plasma aliquots are stable at 2-8 °C for 48 hours. Aliquots may be stored at -20 °C for 2 months. Avoid repeated freezing and thawing. Do not attempt to thaw specimens by heating in a water bath. If specimens are not to be used immediately, cover with Parafilm and place in refrigerator.

3. Reagents, Supplies, and Storage

a. All reagents, standards and controls are stored in the RIA refrigerator at 2-8 °C. Refer to expiration dates on the bottle.

b. 125-I testosterone tracer -- 20 ml of radiolabeled testosterone-3-CMO histamine, less than 4 microcuries. Contains 0.025M phosphate buffer testosterone-binding inhibitor and 0.05% sodium azide.

c. Testosterone antiserum -- 20 ml of rabbit antihuman testosterone antiserum in 0.01M phosphate buffer, pH 7.4, and 0.25% BSA, 1.0 mg/ml lipase, and 0.1% sodium azide.

d. Testosterone standards -- 2.0 ml containing 0, 15, 50, 100, 250, 500, and 1,000 mg/dl of testosterone in human serum with 0.1% sodium azide.

e. PEG second antibody solution -- 100 ml containing 6% PEG (polyethyleneglycol), and goat antirabbit antibody, with 0.05% BSA bovine serum albumin and 0.1% sodium azide.

f. Diluent -- 3 ml containing a stripped human serum base and 0.1% sodium azide.

g. Before testing, all reagents should be at room temperature and should be mixed thoroughly by gentle inversion. These reagents are considered to be potentially infective biological materials and should be handled with caution.

h. 12- X 75-mm polypropylene test tubes
i. Test-tube rack
j. Water bath capable of maintaining 37 °C
k. Accuprep pipetting station
l. Vortex mixer
m. Sorval RC-3 automatic refrigerated centrifuge
n. Isodata gamma counter calibrated to detect 125I

4. Quality Control Material

Two levels of Lyphochek controls, Lyphodil and level II, will be run in duplicate, one level each at the beginning and end of each analytical run.

To reconstitute the product, add 5.0 ml of type I water with a class A volumetric pipette to the desired vials. Replace the rubber stopper and allow the reconstituted material to stand at least 15 minutes. After that time, invert the bottle several times to ensure that the contents are thoroughly mixed. Before initial use, inspect each vial carefully for undissolved material. Reconstituted controls should be stored at 2-8 °C, and they are stable for 7 days.

Reconstitute the Lyphochek controls as above. To the Lyphochek level I add exactly 1.0 ml of Lyphochek level III using a 1-ml class A volumetric pipet. Mix thoroughly and label as "Lyphodil Control."

PROCEDURE

Action	Result
1. Allow all samples, controls, standards, and reagents to come to room temperature. Mix by gentle inversion. Turn water bath on 37 °C.	
2. Label 12- X 75-mm assay tubes in duplicate for each standard, control, participant sample, nonspecific binding, and total count. Dilute all participant samples with test diluent. Use these diluted forms as samples. Note: Before beginning the testosterone procedure, dilute all participant samples with the testosterone diluent. Label 12- X 75-mm assay tubes for diluted samples.	
3. Prime line from channel 2, five times with air.	To remove water from line 5.
4. Place line from channel 2 into testosterone diluent. Prime twice into waste and 10 times back into reagent bottle.	
5. Press, PROGRAM 13, ENTER, on the Accuprep.	This will bring up the TDIL program.
6. Press hand-control switch.	This will pull up 140 ul of diluent into channel 2 and 800 ul of water into channel 1.
7. Press hand-control switch again.	This will pull up 10 ul of air into channel 1.
8. Place probe tip into participant sample.	
9. Press hand-control switch.	150 ul of participant sample will be drawn into channel 1.
10. Place probe tip into labeled tubes for dilution.	
11. Place hand-control switch.	140 ul of participant sample and 140 ul of diluent will be dispensed to make 1:2 dilution of the participant sample.

Action	Result
12. Place probe tip into waste container.	
13. Press hand-control switch.	800 ul of water will rinse tip.
14. Repeat until all tubes are diluted.	
15. When tubes are completed, remove channel 2 line from diluent and place into water.	Prime 20 times to clean.
16. Use diluted samples for assay. Gamma counter will give a corrected value for the dilution.	
17. Remove the Accuprep channel 2 line from water. Press PRIME, CHANNEL, ENTER, RUN.	This will prime the line five times with air to remove water from line.
18. Accuprep channel 1 line should be in water. Place channel 2 line in testosterone tracer.	
19. PRIME channel 2 line 2 times into water and 20 times back into the reagent bottle.	Second primes will remove any residual water from line.
20. Press PROGRAM, 01, ENTER.	This will bring up the TST1 program.
21. Pipet the nonspecific binding tubes.	
a. Press hand-control switch or the run button.	This will pull up 750 ul of water into channel 1 and 200 ul of tracer into channel 2.
b. Press hand-control switch again.	10 ul of air will be drawn into channel 1. (This action separates the wash water from the sample.)
c. Place probe tip into 0 standard.	
d. Press hand-control switch.	105 ul of sample will be drawn into channel 1.
e. Place probe tip in nonspecific binding tube.	
f. Press hand-control switch.	100 ul of sample and 200 ul of trace will be dispensed.
g. Place probe tip in waste.	

Action	Result
h. Press hand-control switch.	Channel 1 will rinse itself with 150 ul of water and remove the extra 5 ul of sample.
i. Repeat step 7 for the second nonspecific binding tube.	
22. Pipet total count tubes.	
a. Press hand-control switch twice.	This will draw up water and air into channel 1 and reagent into channel 2.
b. Let probe tip remain in air.	
c. Press hand-control switch.	Instead of sample, only air will be drawn into channel 1.
d. Place probe tip in total count tube.	
e. Press hand-control switch.	Only tracer will be dispensed into the tube.
f. Place probe tip in waste.	
g. Press hand-control switch.	To rinse line.
h. Repeat step 8 for second total count tube.	
i. Cover these tubes with Parafilm and set aside.	
23. Pipet the standard, control, and participant sample tubes.	
a. Press hand-control switch.	This will pull water and air into channel 1, and tracer into channel 2.
b. Place probe tip in desired standard, control, or participant sample.	
c. Press hand-control switch.	Specimen will be drawn into channel 1.
d. Place probe tip into appropriately labeled tube.	
e. Press hand-control switch.	100 ul of specimen and 200 ul of tracer will be dispensed.
f. Place probe tip in waste.	
g. Press hand-control switch.	To rinse line.
h. Repeat step 19 for all standards, controls, and participant tubes.	

Action	Result
24. When all tubes are complete, EXIT the program.	
25. Before removing channel 1 line from water, press PROGRAM, 02, ENTER.	This will bring up the TST2 program.
26. Complete the nonspecific binding tubes.	
a. Press hand-control switch.	This will pull up 1,000 ul of water into channel 1. (Nonspecific binding tubes receive water instead of antiserum.)
b. Place probe tip into nonspecific binding tubes.	
c. Press hand-control switch.	This will dispense 200 ul of water into each tube.
d. Place the nonspecific binding tubes aside.	
27. EXIT the program.	
28. Remove Accuprep channel 1 line from water and channel 2 line from tracer.	
29. Place channel 1 line into water. PRIME six to seven times into waste.	This will wash the tracer out of the line. More complete cleaning will be done later.
30. PRIME channel 1 line 5 times with air.	This will remove any residual water from line.
31. Place channel 1 line into the testosterone antiserum. PRIME 2 times into waste and 20 times back into reagent bottle.	
32. Press PROGRAM, 02, ENTER.	This will bring up the TST2 program.
33. Press hand-control switch.	1,000 ul of antiserum will be drawn into channel 1.
34. Place probe tip into all assay tubes in turn. Press hand-control switch for each tube.	200 ul of antiserum will be dispensed into each tube. When empty, the syringe will automatically refill.
35. Repeat until procedure is completed for all tubes.	
36. EXIT program.	

Action	Result
37. Mix tubes by vortexing, cover with Parafilm and incubate at 37 °C for 1 hour.	
38. While tubes are incubating, remove channel 1 line from antiserum and place into water.	
39. PRIME 20 times into waste to clean.	
40. Place channel 2 line into Beckman TraceKlean and PRIME 20 times then place into water and again PRIME 20 times.	This line is now clean.
Note: Repeat Eppendorf pipet may also be used to dispense polyethyleneglycol (steps 41-48).	
41. PRIME channel 1 with air five times.	This will remove the water from the line.
42. Place channel 1 line into the polyethyleneglycol second antibody solution.	
43. PRIME channel 1 line 2 times into waste and 20 times back into the reagent bottle.	
44. After incubation, press PROGRAM, 03, ENTER.	This will bring up the TST3 program.
45. Press hand-control switch.	This will pull up 1,000 ul of reagent into channel 1.
46. Place probe tip into each assay tube in turn. Press hand-control switch for each tube.	1,000 ul of reagent will be dispensed into each tube.
47. Repeat until all tubes are complete.	
48. EXIT the program.	
49. Mix contents of tubes by vortexing and spin in Sorval RC-3 centrifuge for 20 minutes at 3,000 rpm and at 2-8 °C.	
50. While tubes are spinning, remove channel 1 line from the polyethyleneglycol and place into water. PRIME 20 times into waste to clean.	

Action	Result
51. After tubes have spun, decant, and blot the rims carefully.	Do not allow tubes to remain inverted for more than 1 minute.
52. Read results from gamma counter. Refer to Isodata operational instructions for specifics.	

6. Reference

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V. ANALYTICAL METHODS

U. T and B Lymphocytes in Serum
Flow Cytometric Fluorescence Determination

1. Principle

The numbers of T and B lymphocytes present in circulating blood are often a useful parameter by which to gauge a person's immune competence to defend, for example, against bacterial and viral infections. Monoclonal antibodies OKT3 (pan T cell) and CCB1 (pan-B cell) can be used to enumerate these two lymphoctye types. Two T cell subsets exist: the helper/inducer subset (bound by OKT4A monoclonal antibody) and the cytotoxic/suppressor subset (bound by OKT8 monoclonal antibody). The relative proportion of each of these two T cell subsets (i.e., the T4/T8 cell ratio) in the circulation has also recently been described as an indicator of immune status.

The various lymphocyte subpopulations are enumerated by staining peripheral blood mononuclear cells with the appropriate fluorescein-tagged monoclonal antibodies, determining the percentage of these cells that are fluorescent by analysis in a flow cytometer and converting these percentages to total cell counts per milliliter of blood. This last calculation is made by multiplying the various lymphocyte subset percentages by the total lymphocytes per milliliter of blood as determined by microscopic differential counts performed on whole blood smears.

2. Specimen

Peripheral blood mononuclear cells (PBMC) are prepared from whole blood. (See procedure.)

3. Reagents, Supplies, and Storage

a. RPMI 1640 tissue culture medium, stock solution
To prepare 5 L:

(1) Allow five 1-L packages of RPMI-1640 powder (Gibco no. 430-1800) to come to room temperature. Dissolve in 500 ml distilled deionized water in a 1,000-ml graduated cylinder, while stirring with a magnetic stir bar.

(2) QS to 1 L with water and transfer to a 5-L flask.

(3) While stirring with a magnetic stir bar, add in this order:

(a) 3,495 ml water
(b) 5 ml gentamyicin (Gibco no. 600-5710, 10 mg/ml stock)
(c) 10 g $NaHCO_3$ powder (Sigma no. S-8875)
(d) 11.9 g HEPES (Sigma no. H-3375, acid form)

Total volume: 4,500 ml

(4) Sterilize by dispensing 450-ml aliquots under positive pressure through a 0.2-u filter into each of 10 sterile 500-ml sterile screwcapped media bottles. Label bottles appropriately.

(5) Store these bottles tightly capped at 4 °C.

(6) Test for sterility. Using sterile technique, transfer 1-ml samples from each bottle to appropriately labeled culture tubes. Incubate these tubes at 37 °C for 1 week. If any turbidity develops in a tube, label the bottles from which that sample came "Non-Sterile" until the medium is refiltered and certified to be sterile.

b. Serum

(1) When the serum is first received (Hyclone fetal bovine serum defined, no. 100438, 500-ml bottle), store it at -20 °C.

(2) Thaw the serum. Make sure the serum is completely thawed and well mixed before proceeding to the next step.

(3) Dispense the serum in 100-ml aliquots into five 100-ml screwcapped bottles. USE STERILE TECHNIQUE to perform these transfers.

(4) Incubate these bottles (tightly capped) 30 minutes in a 56 °C water bath to inactivate complement. Label the bottles "Heat Inactivated."

(5) Store the bottles at 4 °C (for less than 2 weeks) or at -20 °C (for longer than 2 weeks). Use sterile technique when sampling serum.

(6) Test for sterility. Using a sterile technique, transfer 1-ml samples from each bottle to appropriately labeled culture tubes. Incubate these tubes at 37 °C for 1 week. If any turbidity develops in a tube, label the bottle from which that sample came "Non-Sterile", and store it at -20 °C.

c. RPMI-0% (not supplemented with serum)

(1) To 450 ml of RPMI-1640 stock solution, add 50 ml of sterile distilled deionized water.

(2) Store at 4 °C. Discard if turbidity develops.

d. RPMI-5% (supplemented with 5% serum)

(1) To 450 ml of RPMI-1640 stock solution, add:

(a) 25 ml sterile distilled deionized water.
(b) 25 ml serum.

(2) Store at 4 °C. Discard if turbidity develops.

e. PBS 1X (Phosphate-buffered saline without Ca++ or Mg++)

(1) Store 10X stock solution (Gibco no. 310-4200) in 500-ml bottle at room temperature.

(2) To make use the dilution (1X), add:

(a) 50 ml PBS 10X stock
(b) 450 ml distilled deionized water

(3) Store use dilution (1X) at 4 °C.

f. Suspension buffer (PBS + 5% serum + 0.1% sodium azide)

(1) To make suspension buffer add:

(a) 475 ml PBS 1X
(b) 25 ml serum
(c) 0.5 g sodium azide (Sigma no. S-2002)

(2) Store at 4 °C. Discard if turbidity develops.

g. Ficoll-Paque (Pharmacia no. 17-0840-03). Store at 4 °C in an amber bottle or otherwise protected from light.

h. Fluorescent microsphere alignment standards (beads) (Coulter no. 6602773, 10 um Fullbright Grade I, CV=1.5%) Store at 4 °C. Protect from light.

i. Propidium iodide

(1) To make 200 ug/ml (100X) Stock Solution: Dissolve 4 mg propidium iodide (Sigma no. P-5264) in 20 ml PBS 1X.

(2) Dispense in aliquots of 400 ul into 500 ul conical polypropylene microfuge tubes and store at -80 °C.

(3) On day of use, to make 10X use solution: Thaw one vial of stock solution and dilute with 3.6 ml of PBS 1X.

j. Monoclonal antibodies (FITC-conjugated)

(1) Reconstitute each bottle of antibodies as follows:

(a) Add 1 ml distilled deionized water to:

IgG2a control (Ortho no. 7200, 50 ug)
OKT3 (Ortho no. 7031, 50 ug)
OKT4A (Ortho no. 7041, 50 ug)
OKT8 (Ortho no. 7081, 25 ug)

(b) Add 0.5 ml distilled deionized water to:

(i) CCB1 (Coulter no. 6602381, 2,000 ug)

(2) Invert bottle several times until antibodies go into solution.

(3) Allow to equilibrate at room temperature for 5-10 minutes.

(4) Dilute the antibodies to a concentration of 25 ug/ml by adding the following amounts of suspension buffer:

(a) Add 1 ml buffer per 1 ml antibody:

(i) IgG2 control
(ii) OKT3
(iii) OKT4A

(b) DO NOT dilute OKT8 (concentration is already 25 ug/ml)

(c) Add 39.5 ml buffer per 0.5 ml antibody: CCB1

(5) Dispense 250 ul aliquots of diluted antibody preparations into 500-ul polypropylene microfuge tubes, properly labeled with identification, lot number, date. [Ab] = 25 ug/ml.

(6) Store these tubes, tightly capped at -80 °C.

(7) For use, thaw one tube of each type of antibody. In a 1.5-ml poylpropylene microfuge tube combine:

(a) 375 ul suspension buffer
(b) 250 ul thawed antibody preparation
[Ab] = 10 ug/ml

(8) Allow to equilibrate at room temperature 5-10 minutes before using. This should be sufficient antibody for 125 tests. Be certain there is enough antibody to stain all the samples for any given day; if not, prepare a whole new set of antibodies.

(9) Store use dilution of antibodies at 4 °C and protect from exposure to light.

4. Quality Control Material

Quality control of T and B lymphocyte assays requires fresh daily blood specimens. Therefore, a pool of people has been established to give blood once a week. Each run day, specimens will be drawn from two of these persons and assayed in duplicate in each analytic run.

5. Procedure

a. Isolate peripheral blood mononuclear cells (PBMC).

(1) Collect blood by venipuncture, using a 7- or 10-ml green top Vacutainer tube containing sodium heparin.

(2) Quickly (within 2-3 minutes of drawing blood) transfer 5 ml of the blood to a 15-ml polypropylene tube containing 4 ml of room-temperature RPMI-0%. Cap and mix well by inverting the tube several times. Maintain the tube at room temperature with a minimum exposure of light until the next step.

Note: Manipulations of samples from this point forward are performed under the biological safety cabinet, with gloves.

(3) Using a bottle-top dispenser and Pasteur pipet assembly, slowly underlayer the blood-RPMI mixture with 4 ml of room-temperature Ficoll-Paque. Be careful not to disturb the interface.

(4) Centrifuge 30 minutes at 450 X g, 24 ^{o}C, with the centrifuge brake off.

(5) Aspirate supernatant down to about 1 cm above the Ficoll-Paque-medium interface.

(6) Collect cells at the interface with a 5-ml pipet. Collect as little of the Ficoll-Paque as possible; go no more than 1 cm into the Ficoll-Paque layer.

(7) Transfer cells to a separate 15-ml polypropylene tube containing 10 ml room-temperature RPMI-5%. Cap and mix well by gently inverting the tube several times.

(8) Centrifuge the cells 15 minutes at 450 X g, 24 ^{o}C.

(9) Aspirate the supernatant to the notched mark encircling the tube near the tube bottom. The final volume will be about 0.8 ml. Gently resuspend the cells.

10. Transfer the cell suspension to a 12- X 75-mm snapcap polypropylene tube, using a 1 ml pipet. Remove any visible aggregated material from the cell suspension at this time. Immerse the tube in ice and maintain it at 0-4 °C throughout the remainder of the processing.

    Save unused cells from this suspension and store overnight at 0-4 °C; the cells can be used for repeat staining. If repeat staining is not required, the cells may be discarded.

b. Cell counts

    (1) Transfer 125 ul of the cell suspension to a 1.5-ml polypropylene microfuge tube containing 500 ul of PBS and mix.

    This tube will be transported to the clinic on ice for automated determination of cell concentration. When cells have been counted, values will be reported back to the flow cytometry laboratory over a computer link. If the reported value is greater than 5.5 K/ul (Coulter reading), the sample will be restained by using a 7-ul rather than a 35-ul cell suspension in the staining reaction.

c. Cell staining

    (1) Specimens will be stained in groups of eight at a time. One 96 V-bottom wells polystyrene microtiter plate (microplate) (American Scientific Products no. B1190-21) will be used per four specimens; thus, two microplates will be processed at a time.

    (2) Using a micropipetter, transfer 35 ul of cell suspension into each of five V-bottom wells in the appropriate vertical column of a microplate. The microplates and tubes of the cell suspension will be supported on a surface of ice. Cover the microplates with plastic lids.

    (3) Attach each covered microplate atop a rigid carrier 96-well microplate.

    (4) Centrifuge the microplates for 5 minutes, 650 X g, 4 °C.

    (5) Remove the plastic lid. Quickly invert the microplate and flick it downward vigorously three to four times to remove the supernatants. With a paper towel, blot away any supernatant remaining on the plate surface exterior to the wells. Cells remain behind trapped in a volume of approximately 5 ul which is retained at the well bottom by capillary forces.

(6) Detach the flexible microplate from the rigid carrier microplate and resuspend the cells by strumming the bottoms of the V-wells with a fingernail.

(7) Place the covered microplate back on crushed ice. (Keep plates covered whenever possible to avoid contamination.)

(8) To wells in rows 1, 3, 5, 7, and 9, add 5 ul of 10 ug/ml of IgG2a control, OKT3, OKT4, OKT8, and CCB1 antibodies, respectively.

(9) Cover the plate with the plastic lid. Strum the bottom of the microplate to mix the cells and antibodies. Gently but quickly invert the plate; the entire volume will remain trapped in the V-shaped well bottom. Incubate the microplates for 30 minutes at 4 ^{o}C in the dark.

(10) Turn the microplates upright again and place on crushed ice.

(11) Using an 8-channel Finnpipette with pipet tips in every other channel, resuspend the cells, one row at a time, in 200 ul ice cold suspension buffer by pipetting up and down six to eight times. Immediately transfer these suspensions to 500-ul polyproplyene microfuge tubes suspended in trays and supported on ice. Cover with plastic lids. Store on ice and protect from light before flow analysis.

d. Flow cytometric analysis

(1) Turn on the flow cytometer and allow the laser to warm up for at least 2 hours.

(2) Peak laser power after warm-up. Record mwatts and current.

(3) Set mwatts at 500 and amps at 25.00.

(4) Set red-green fluorescent subtraction to 12.0%

(5) Prepare alignment standard by adding approximately 20 ul (two drops) of fluorescent microspheres (Reagent 8) to 500 ul PBS 1X (Reagent 5).

(6) Run alignment standard using program BEADS 5610 and make appropriate alignment adjustments to the flow cytometer. Run "beads" every 20 samples and check the alignment.

(7) Using an Eppendorf repeater pipet, add 20 ul of propidium iodide 10X use solution (Reagant 9) to 20 cell preparations to be analyzed (i.e., one microplate of cell preparations). Allow these samples to incubate at room temperature for 5 minutes to permit nonviable cells to takeup stain, then return cells to ice.

(8) Analyze the 20 propidium iodide-stained samples using program NOREDWIN.

(9) Store histograms on the Winchester hard disc as follows:

Sample	Histograms Stored
(a) Beads:	H4:(90LS x FALS) H1:(GFL) H3:(RFL)
(b) IgG2a:	H1:(L90LS x FALS) H2:(LGFL) H3:(LRFL)
(c) OKT3:	H2:(LGFL)
(d) OKT4:	H2:(LGFL)
(e) OKT8:	H2:(LGFL)
(f) CCB1:	H2:(LGFL)

(10) Repeat steps 6 through 9 until all samples have been analyzed.

6. Calculations

a. "Relative" results are read from the flow cytometer and are reported as "%."

b. Absolute values (in K/mm^3) are calculated as follows:

$$\text{T Lymph/Absolute} = \frac{(1)+(2)}{100} \times (3) \times \frac{\text{T Lymph/Relative}}{100}$$

$$\text{B Lymph/Absolute} = \frac{(1)+(2)}{100} \times (3) \times \frac{\text{B Lymph/Relative}}{100}$$

$$\text{T4 Lymph/Absolute} = \frac{(1)+(2)}{100} \times (3) \times \frac{\text{T4 Lymph/Relative}}{100}$$

$$\text{T8 Lymph/Absolute} = \frac{(1)+(2)}{100} \times (3) \times \frac{\text{T8 Lymph/Relative}}{100}$$

Where:

(1) = lymphocytes
(2) = atypical lymphocytes
(3) = white blood cells

c. The Lymphocyte T4/T8 ratio is calculated as follows:

$$\frac{\text{Lymphocyte T4/Relative}}{\text{Lymphocyte T8/Relative}}$$

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V. ANALYTICAL METHODS

V. Immunoglobulin Levels (IgG, IgA, IgM) in Serum Beckman Immunochemistry System

1. Principle

In this procedure a nephelometer is used; it measures the rate of light scatter formation from an immunoprecipitin reaction. Anti-IgG, anti-IgA, and anti-IgM, when brought into contact with their respective antigens in solution, produce a peak rate signal proportional to the increase in light scatter produced by the antigen-antibody reaction.

2. Specimen

The optimum sample size is 250 ul.

Freshly drawn serum from fasting subjects is preferred, but samples may be stored at 2-8 °C for up to 72 hours. Hemolyzed or icteric samples have NOT been shown to interfere with the nephelometric determination of these specific proteins. Lipemic specimens may cause erroneous results.

3. Reagents, Supplies, and Storage

a. Buffer: Phosphate buffered saline with a polymer enhancer and 0.1% sodium azide as a preservative. Use as is. Store at room temperature. Note: Reagent must be warmed to room temperature before use. Usable until expiration date on bottle.

b. Diluent: Phosphate buffered saline with 0.1% sodium azide as a preservative. Use as is. Store at room temperature. Usable until expiration date on bottle.

c. Calibrator: 3.0 ml processed human serum containing IgG IgA, and IgM at a concentration near the midpoint of the measuring of the test result. Contains 0.1% sodium azide as a preservative. Store at 2-8 °C. Warm to room temperature before use.

d. Antiserum to human IgG, IgA, and IgM: Each bottle contains 5.0 ml of the respective antibody with 0.1% sodium azide as a preservative. Each antiserum incorporates a blue dye which signals the analyzer that the antigen-antibody reaction has been started. Store at 2-8 °C. Warm to room temperature before use. Usable until expiration date on bottle.

4. Calibration

The Auto ICS is calibrated at a single calibrator concentration for each protein defined by using constants provided by the antibody

card, the target value of the calibrator serum on the calibrator card, and the appropriate dilution of calibrator serum. The constants provided by the antibody card are values that represent the concentration/rate relationship throughout the measuring range. The analyzer electron system uses these data to characterize a curve-fitting formula that yields a mathematically unique curve for each bottle of antibody.

Calibration is done by testing a single protein concentration contained in a specific dilution of calibrator serum in duplicate. The peak rate signal obtained during calibration (the raw calibrator value) is used to establish a ratio to the peak rate expected on the basis of the assigned calibrator serum target value. The calibration factor is used to adjust the analyzer electronics system gain so that the raw calibration values equal the target value specified on the calibrator card. All subsequent sample rate signals are similarly adjusted.

To ensure a valid calibration, the analyzer electronics system requires that the peak rate measurement obtained during calibration be reproduced within a predefined percentage (typically ±5%) in two of two measurements or two in a series of three measurements. When the first and second peak rate values fall within the required range, the two values are averaged, and the peak rate signal is internally adjusted so that the calibrator will read at its target value.

5. Quality Control Material

   a. Normal pool: Obtain one to two bags of plasma from blood bank.

   b. Abnormal pool: Obtain bags of plasma of a known monoclonal gammopathy patient (blood services). Dilute with normal plasma, if necessary, to bring values into a workable range.

   c. Preparation: Recalcify by adding one part of 2.77% solution of calcium chloride (made in distilled water) to nine parts of plasma. Incubate at 37 °C for 2 hours. Freeze overnight at -70 °C. Pour off the serum from the retracted clot. Add sodium azide to equal 0.1% concentration as a preservative. Aliquot in 0.5 ml volumes in microcentrifuge tubes. Store at -70 °C. Thaw the vials and allow them to reach room temperature before use. Mix well before testing.

   d. Run each control in duplicate, one placed at the beginning and one placed randomly at a later position in each analytical run.

6. Procedure

   a. Allow the calibrator serum and antiserum to come to room temperature before use. (Allow at least 30 minutes.)

b. Perform daily maintenance and startup procedure. (See maintenance schedule in the operation manual.)

c. When SELECT LIT FUNCTION KEY appears on the data processor display, press the PROT key.

d. Enter the date, operator's initials, and comments when prompted. (If no comment is needed, press ENTER.)

   Terminate each entry by pressing the ENTER key after each input.

e. Insert the antibody card into the reader when display reads READ AB CARD FOR WELL 1. Remove the card and place the vial in position 1. Remove the cap and replace with reagent cover. Continue this procedure until all antibody cards have been read. Press END key.

f. Insert the calibrator card when prompted by the display and remove the card.

g. Put two 2.0-ml sample cups in the special dilution wells.

h. Place 250 ul of calibrator serum into a 0.5-ml sample cup and place it on the turntable in the cup location specified by the data processor printout (cup no. 40).

i. Place a minimum of 150 ul of each test sample and 120 ul of control sample into 0.5-ml sample cups and place the cups in the desired positions on the turntable.

j. Place the necessary number of dilution segments on the turntable.

k. The data processor display will show (EDIT, END): ENT CUP NO:1. Press THRU key followed by the number of the last cup for that day's run. Press ENTER; data processor display will show 1T (#) ENTER AB NO. Press ALL. Press ENTER.

l. When cup and antibody definitions are complete, press I. Display will read, ID: (ENT) or ENT CUP: 1. Press ENTER. Enter sample ID number for cup 1 and read CUP-NO. 2 ID=?. Enter sample ID, press ENTER, and continue for the remainder of the samples. Press END when finished with ID programming; press END again to exit sample programming.

m. End of run is signified by beeping.

7. Results

The immunochemistry analyzer will automatically report the results in milligrams per deciliter. If replicates are measured, the technologist, using the computer, must average the two measurements. The two measurements should agree within 5% Results are reported as whole numbers.

8. References

a. Beckman Instruments. Automated immunochemistry system operating manual. Brea, California: Beckman Instruments, 1983.

b. Beckman Instruments. ICS immunoglobulin panel reagent test kit [package insert]. Brea, California: Beckman Instruments, 198[illegible].

V. ANALYTICAL METHODS

W. Hepatitis B Surface Antigen (HBsAg) in Serum

1. Principle

Beads coated with mouse monoclonal antibody to hepatitis B surface antigen (anti-HBs) are incubated with serum or plasma, appropriate controls, and mouse monoclonal anti-HBs peroxidase (horseradish) conjugate (anti HBs:HRPO). During the incubation, any HBsAg present is bound to the solid phase antibody and simultaneously bound by the anti-HBs:HRPO. Unbound material is aspirated and the beads are washed.

Next, O-phenylenediamine (OPD) solution containing hydrogen peroxide is added to the bead. After incubation, a yellow-orange color develops in proportion to the amount of HBsAg bound to the bead.

The enzyme reaction is stopped by the addition of acid. The absorbance of the controls and of the participant specimens is determined by using a spectrophotometer with the wavelength set at 492 nm. Specimens with absorbance values equal to or greater than the absorbance value of the negative control mean, plus a factor, are considered reactive for HBsAg.

Specimens nonreactive by the Auszyme monoclonal tests are considered negative for HBsAg and need not be tested further. A specimen found to be reactive should be confirmed as being reactive by neutralization procedures in which human anti-HBs (HBsAg monoclonal confirmatory assay) are used.

2. Specimen

Either serum or plasma may be used. Stored specimens should be refrigerated at 2-8 °C. For long-term storage, the specimens should be frozen (at -15 °C or colder).

3. Reagents, Supplies and Storage

Note: Reagents a-f are stored at 2-8 °C and must be warmed to room temperature (18-30 °C) before use.

a. Anti-HBs monoclonal coated beads: Antibody to hepatitis B surface antigen (mouse monoclonal).

b. Auszyme monoclonal conjugate: Antibody to hepatitis B surface antigen (mouse monoclonal). Peroxidase (horseradish). Minimum concentration: 0.2 ml in TRIS buffer with protein stabilizers. Preservative: gentamicin sulfate and thimerosal. Dye: red, no. 33.

c. Positive control: Human HBsAg, 9 ±2 ng/ml in TRIS buffer with protein stabilizers. Preservative: gentamicin sulfate and thimerosal. Dye: bromophenol blue.

d. Negative control: Recalcified human plasma, nonreactive for HBsAg and Anti-HBs. Preservative: gentamicin sulfate and thimerosal.

e. OPD (O-phenylenediamine.2 HCl) tablets, 12.8 mg.

f. Diluent for OPD. Citrate-phosphate buffer containing 0.02% hydrogen peroxide.

g. 1N sulfuric acid: The stopping reagent is provided as an accessory to the Auszyme monoclonal kit. Store at room temperature (18-30 °C). To be suitable as a stopping reagent, the sulfuric acid in each new lot number must pass the following test:

(1) Pipet 300 ul OPD substrate solution into five reaction tubes.
(2) Add 2 ml of 1N H_2SO_4 being checked to each tube.
(3) Use distilled water as a blank.
(4) Measure the absorbance of the OPD/acid solution at "0 time" and "120 minutes," going into the "Cal" mode on the Quantamatic, using the linearity program.
(5) "0 time" absorbance must be less than 0.08.
(6) The difference between the values obtained at "0 time" and "120 minutes" must be less than 0.03 units.

h. Sodium hypochlorite (add 500 ml Clorox to a waste container for a final mixture of 2.5% sodium hypochlorite). Store at room temperature.

i. 5% sodium hypochlorite (50 ml. Clorox to 1 L). Use this solution for wiping spills. Store at room temperature.

j. Lab-prepared 1N H_2SO_4 (49 ml H_2SO_4 + 951 ml H_2O). Use this solution to keep all dispensers and bottles scrupulously clean. Store at room temperature.

4. Quality Control Material

Three negative controls and two positive controls are assayed with each analytical run.

Individual negative control values should be between -0.006 and 0.100. They should also be between 0.5 and 1.5 times their mean.

The positive control mean minus the negative control mean must be greater than 0.200.

5. Procedure

a. Bring all reagents to room temperature. (18-30 °C). Mix gently before using.

b. Turn on water bath (40 °C ± 1 °C).

c. Label reaction wells.

Position 1 = Blank
Position 2, 3, 4 = Negative
Position 5, 6 = Positive
Position 7, etc. = Participant samples, run in duplicate

d. Pipet 50 ul of conjugate into each reaction well, except no. 1, the blank.

e. Pipet 200 ul of control or participant specimens into their respective wells.

f. Mix by gently tapping the side of the tray. DO NOT SPLASH!

g. Add one bead to all control and participant sample wells. To the blank well, add a pink filler bead. Also add filler beads to any incomplete rows.

h. Gently tap the side of the tray to remove trapped air. Beads must be immersed in the liquid. DO NOT SPLASH!

i. Cover with a paper seal. Label with the test name, date, and incubation finish time.

j. Float in water bath (40±1 °C) for 75 minutes.

k. Five to ten minutes before the end of incubation, make up OPD reagent. Reconstituted OPD must be used within 60 minutes.

(1) Add four OPD tablets to the designated brown bottle with the plastic forceps provided.
(2) Add 20 ml of OPD diluent to the bottle. Let the solution sit until tablets dissolve.
(3) Mix well. Let the solution sit to get rid of bubbles.
(4) Mix gently before applying the 300-ul dispenser to ensure homogenicity.
(5) Apply the dispenser top.
(6) Immediately before dispensing, prime five times into waste, then prime back into bottle.

l. Remove tray from the water bath. Wash each well with 15 ml distilled water.

(1) Place the tray in a riser rack on the washing track.
(2) Enter: the number of wells to be processed (1-999, divisible by 5, e.g., 50)
(3) Press: WASH
(4) Enter: milliliters of water (15)
(5) Press: GO
(6) After the gears start to turn, GENTLY push the rack to connect with the gears.

m. Immediately invert the tray into the box of tubes. Match the A-1 position on the tray with the A-1 position in the box. Tap to drop all beads into the tube. Remove the pink bead from blank tubes.

n. Place the box on the rack and place on the dispensing track.

o. Place OPD reagent in position 2 of Pro-Quantum.

p. Add 300 ul of OPD to each tube, including the blank.

(1) Enter: number of wells to be processed (exact number, e.g., 48)
(2) Press: DISPENSE
(3) Enter: Station number (2)
(4) Press: GO

q. Cover and incubate for 30±2 minutes at room temperature. (18-3[illegible] °C).

r. Place the 1N sulfuric acid bottle with the 1-ml dispenser in position 3 on the Pro-Quantum.

s. After 30 minutes, place the box of tubes on a rack on the dispenser track and add the sulfuric acid to each tube as in step p.

t. Agitate the tubes and remove the air bubbles.

u. Read on the Quantumatic, program 2 within 2 hours after the addition of H_2SO_4. (See section on using the Quantumatic in the Operator's Manual.) Read blank only with H_2SO_4 and OPD.

7. Results

a. Determine the cutoff value.

b. Add 0.025 to the negative control absorbance mean.

c. A participant sample absorbance value less than the cutoff valu[illegible] is reported as negative.

d. A participant sample absorbance value greater than the cutoff value is reported as positive only if the result is confirmed b[illegible] neutralization.

e. A participant result found to be negative by the confirmatory method is reported as negative.

f. A participant result that falls within 10% of the cutoff value is reported as borderline negative or borderline positive as long as the two readings are similar.

g. If the two readings on borderline values do not agree, the sample should be retested.

8. References

a. Abbott Laboratories Diagnostic Division. Antibody to hepatitis B surface antigen (mouse monoclonal): peroxidase (horseradish) conjugate auszyme monoclonal: qualitative third generation enzyme immunoassay for the detection of hepatitis B surface antigen (HBsAg) in human serum or plasma. North Chicago, Illinois: Abbott Laboratories Diagnostic Division, 1984.

b. Abbott Laboratories Diagnostic Division. Pro-Quantum bead washer and reagent dispenser operator's manual. North Chicago, Illinois: Abbott Laboratories Diagnostic Division, 1984.

V. ANALYTICAL METHODS

X. Antibody to Hepatitis B Core Antigen (HBcAb) in Serum
Abbott Enzyme Immunoassay

1. Principle

In the Corzyme enzyme immunoassay, beads coated with HBcAg are incubated with serum or plasma and human anti-HBc conjugated with horseradish peroxidase (anti-HBc:HRPO). After incubation, the unbound material is removed by washing the bead. If anti-HBc is present in the specimen, it will compete with anti-HBc:HRPO for a limited number of HBcAg binding sites on the bead.

Next, O-phenylenediamine (OPD) solution containing hydrogen peroxide is added to the bead, and after incubation, a yellow color develops in proportion to the amount of anti-HBc:HRPO that bound to the bead during the previous incubation. The enzyme reaction is stopped by adding 1N sulfuric acid. The absorbance of controls and specimens is determined by using a spectrophotometer with wavelength set at 492 mn. Within limits, the greater the amount of anti-HBc in the specimen, the lower the absorbance.

With acute hypatitis B infection, anti-HBc is found in serum shortly after hepatitis B surface antigen (HBsAg) appears and, after HBsAg disappears, it persists until detectable antibody to the HBsAg (anti-HBs) appears. Therefore, in the absence of HBsAg and anti-HBs, anti-HBc may be the only serological marker of recent hepatitis B infection and potentially infectious blood.

2. Specimen

The Corzyme test may be performed on human serum or plasma.

a. Specimens are stored in the refrigerater at 2-8 °C or frozen.

b. Specimens containing sodium azide should not be tested because they may give false positive reactions.

3. Reagents, Supplies, and Storage

Note: Reagents a-f are stored at 2-8 °C. All must be warmed to room temperature (18-30 °C) before use.

a. Hepatitis B core antigen (human) coated beads.

b. Antibody to hepatitis B core antigen (human): Peroxidase (horseradish) conjugate. Conjugate concentration minimum: 0.2 ug/ml. Preservative: 100 ug/ml gentamicin sulfate, USP.

c. Positive control (recalcified human plasma, positive for anti-HBs and anti-HBc). Anti-HBc, minimum titer 1:200 ±2 log 2 dilutions. Preservative: 100 ul/ml gentamicin sulfate USP.

d. Negative control (recalcified human plasma, nonreactive for HBsAg, anti-HBs, and anti-HBc). Preservative: 100 ug/ml gentamicin sulfate, USP.

e. OPD (O-phenylenediamine.2HCl) tablets.

f. Diluent for OPD: Citrate-phosphate buffer containing hydrogen peroxide.

g. 1N sulfuric acid. Store at room temperature (18-30 °C).

If sulfuric acid is made inhouse:

9.8 ml	conc H_2SO_4
+190.2 ml	water
200.0 ml	Total

Each time the sulfuric acid solution is made, it must pass the following test. If it does not, sulfuric acid from a new lot must be used to prepare a new solution.

(1) Pipet 300 ul of OPD substrate solution into five reaction tubes.
(2) To each tube, add 2 ml of the 1N H_2SO_4 that is being checked.
(3) Use distilled water as a blank.
(4) Measure the absorbance of the OPD/acid solution at "0 time" and "120 minutes," going into the CAL mode on the Quantumatic, using the linearity program.
(5) "0 time" absorbance must be less than 0.08.
(6) Difference in the values obtained at "0 time" and "120 minutes" must be less than 0.03 units.

h. Sodium hypochlorite: Add 500 ml Clorox to waste container for a final mixture of 2.5% sodium hypochlorite. Store at room temperature.

i. 5% sodium hypochlorite: Add 50 ml Clorox to 1L. Use for wiping spills. Store at room temperature.

j. Lab-prepared 1N H_2SO_4 (49 ml conc. H_2SO_4 + 951 ml H_2O). Use this solution to keep all dispensers and bottles scrupulously clean. Store at room temperature.

4. Quality Control Material

Three negative and two positive controls must be assayed with each analytical run. All controls and participant samples must be subjected to identical incubation times and handling.

- The values for the negative controls should fall between 0.5 and 1.5 times their mean.
- Their absorbance must be larger than that of the positive controls.
- The positive control absorbance mean subtracted from the negative control absorbance mean must be greater than 0.3. If it is not, the run should be repeated because the techniques may have been faulty or the reagents may have deterioriated.

5. Procedure

a. Bring all reagents to room temperature (18-30 °C). Mix gently before using.

b. Turn on water bath (40±1 °C).

c. Label reaction wells.

Position 1 = blank
Position 2, 3, 4 = negative
Position 5, 6 = positive
Position 7, etc. = participant samples, run in duplicate

d. Place antibody with 200-ul dispenser top into position 1 of Pro-Quantum.

e. Dispense 200 ul of antibody into reaction wells:

(1) Position the riser rack on the dispensing track.
(2) Enter: the number of wells to be skipped (use for blank, skip 1)
(3) Press: SKIP
(4) Enter: the number of wells to be processed (exact number, e.g., 48)
(5) Press: DISPENSE
(6) Enter: Station number (1)
(7) Press: GO

f. Pipet 100 ul of control and participant specimens into their respective wells.

g. Tap the edge of tray vigorously to mix. DO NOT SPLASH!

h. Add one bead to all control and participant wells. To the blank well, add a pink filler bead. Also add filler beads to any incomplete rows.

i. Gently tap the side of the tray to remove trapped air. Beads must be immersed in the liquid. DO NOT SPLASH!

j. Cover with paper seal. Label with test name, date, and incubation finish time.

k. Float in water bath (40±1 °C) for 2 hours ±5 minutes.

l. Five to ten minutes before end of incubation make up OPD reagent (reconstituted OPD must be used within 60 minutes).

(1) Add required number of OPD tablets to labeled OPD brown bottle according to the number of wells used:

No. of Wells	No. of Tablets	Diluent Vol.
13	1	5 ml
28	2	10 ml
43	3	15 ml, etc.

(2) Add designated volume of diluent to the bottle. Let the bottle sit until the tablets dissolve.
(3) Mix well. Let sit to get rid of bubbles.
(4) Mix gently before applying 300 ul dispenser to ensure homogenicity.
(5) Apply dispenser top.
(6) Immediately before dispensing, prime five times into waste and then prime back into bottle.

m. Remove the tray from the water bath. Wash each well with 15 ml distilled water:

(1) Place the tray in a riser rack on the washing track.
(2) Enter: the number of wells to be processed (1-999, divisible by 5, e.g., 50)
(3) Press: WASH
(4) Enter: milliliters of water (15)
(5) After gears start to turn, GENTLY push the rack to connect with the gears.

n. Immediately invert tray into the box of tubes. Match the A-1 position on the tray with the A-1 position in the box. Tap to drop all beads into the tubes. Remove the pink bead from blank tubes.

o. Place the box on the rack and place the rack on the dispensing track.

p. Place OPD reagent in position 2 of Pro-Quantum.

q. Add 300 ul of OPD to each tube as in step e, except that OPD is also added to the blank. (Use position 2.)

r. Cover and incubate for 30±2 minutes at room temperature (18-30 °C).

s. Place the 1N sulfuric acid bottle with the 1-ml dispenser in position 3 on the Pro-Quantum.

t. After 30 minutes place the box of tubes on a rack on the dispenser track and add the sulfuric acid to each tube, including the blank, as in step e.

u. Agitate the tubes and remove the air bubbles.

v. Read on the Quantumatic, program 3 within 2 hours after the addition of sulfuric acid. (See section on using the Quantumatic.) Read blank containing only sulfuric acid and DIH_2O.

6. Results

The presence or absence of anti-HBc is determined by comparing the absorbance of the specimen to a cutoff value. This cutoff value is calculated from the negative control mean (NCx) and positive control mean (PCx) absorbance.

Unknown specimens with an absorbance value equal to, or lower than, the cutoff value are considered reactive for anti-HBc.

Those specimens with an absorbance value higher than the cutoff value are considered negative for anti-HBc.

Specimens with absorbance values within 10% of the cutoff value are reported borderline negative or borderline positive as long as the two readings agree. (Duplicate participant values that do not agree should be repeated.)

Determination of cutoff value = 0.4 (negative absorbance mean) + 0.6 (positive absorbance mean) = cutoff value.

7. References

a. Abbott Laboratories Diagnostic Division. Corzyme enzyme immunoassay for the detection of antibody to hepatitis B core antigen in serum or plasma. North Chicago, Illinois: Abbott Laboratories Diagnostic Division, 1984.

b. Abbott Laboratories Diagnostic Division. Pro-Quantum bead washer and reagent dispenser operator's manual. North Chicago Illinois: Abbott Laboratories Diagnostic Division, 1984.

V. ANALYTICAL METHODS

Y. Antibody to Hepatitis B Surface Antigen (HBsAb) in Serum

1. Principle

The Ausab EIA test for anti-HBsAg is based on the "sandwich principle," a solid-phase, enzyme-linked immunoassay technique, that measures anti-HBs levels in serum or plasma. Polystyrene beads coated with human hepatitis B surface antigen (HBsAg) are incubated with either the participant specimen or the appropriate control samples.

During incubation, antibody, if present, is immunologically coupled to the solid-phase antigen. After the unbound material is aspirated and the bead is washed, human antigen, tagged with biotin (B-HBsAg) and avidin conjugated with horseradish peroxidase (A-HRPO), is incubated with the antigen-antibody complex on the bead. The biotinylated surface antigen binds to the antibody, creating an antigen-antibody-antigen "sandwich" bead via an avidin-biotin bridge, collectively. Unbound conjugates are aspirated and the beads are washed. Next, O-phenylenediamine (OPD) solution containing hydrogen peroxide is added to the bead, and after incubation, a yellow color develops in proportion to the amount of anti-HBs bound to the bead. Within limits, the greater the amount of antibody in the sample, the higher the absorbance. The enzyme reaction is stopped by the addition of acid. The absorbance of controls and specimens is determined by using a spectrophotometer with wavelength set 492 nm. Specimens with absorbance values equal to or greater than a designated cutoff value are considered reactive for anti-HBs.

Testing for anti-HBs can be useful for (1) evaluating the recovery and prognosis of participants infected with hepatitis B virus (HBV), (2) screening for potential vaccine recipients, and (3) assessing epidemiologic factors associated with the transmission of HBV. The detection of anti-HBs indicates previous exposure to the antigen or vaccine.

2. Specimen

a. The Ausab EIA test may be performed on human serum, plasma, or recalcified plasma.

b. If specimens are to be stored, they should be refrigerated at 2-8 °C or frozen at -15 °C or colder.

c. Specimens containing precipitate may give inconsistent test results. To prevent this problem, clarify such specimens before assaying. (DO NOT USE HEAT-INACTIVATED SPECIMENS.)

Reagents, Supplies, and Storage

Note: For best results, dispense reagents a-g while cold and return to 2-8 °C storage as soon as possible. The exception is that OPD tablets must be warmed to room temperature before the containers are opened. Reconstituted OPD must be held at room temperature and used within 60 minutes.

a. Polystyrene beads coated with hepatitis B surface antigen (human, subtypes ad and ay).

b. Hepatitis B surface antigen (human, subtypes ad and ay): Biotin conjugate. Minimum concentration 0.33 ug/ml in phosphate buffer with protein stabilizers. Preservative: 0.01% gentamicin sulfate.

c. Avidin: peroxidase (horseradish) conjugate. Minimum concentration 0.2 ug/ml in phosphate buffer with protein stabilizers. Preservative: 0.01% gentamicin sulfate.

d. Negative control (recalcified normal human plasma, nonreactive for anti-HBs and HBsAg). Preservative: 0.1% sodium azide.

e. Positive control (recalcified normal human plasma, reactive for anti-HBs). Minimum titer 1:5. Preservative: 0.1% sodium azide.

f. OPD (O-phenylenediamine.2HCl) tablets, 12.8 mg. Store at 2-8 °C.

g. Diluent for OPD: citrate-phosphate buffer containing 0.02% hydrogen peroxide. Store at 2-8 °C.

h. 1N sulfuric acid

The stopping reagent is provided as an accessory to the Ausab EIA diagnostic kit.
Store at room temperature (18-30 °C).
The sulfuric acid in each new lot number must pass the following test:

(1) Pipet 300 ul OPD substrate solution into five reaction tubes.
(2) To each tube, add 2ml of 1N H_2SO_4 being checked.
(3) Use distilled water as a blank.
(4) Measure the absorbance of the OPD/acid solution at "0 time" and "120 minutes," going into the CAL mode on the Quantumatic, using the linearity program.
(5) "0 time" absorbance must be less than 0.08.
(6) The difference in the values obtained at "0 time" and "120 minutes" must be in less than 0.03 units.

Note: Any OPD reagent lot or sulfuric acid lot may be used with any lot of Ausab EIA.

i. Sodium hypochlorite (add 500 ml Clorox to waste container for a final mixture of 2.5% of sodium hypochlorite). Store at room temperature.

j. 5% sodium hypochlorite (50 ml Clorox to 1 L). Use for wiping spills. Store at room temperature.

k. Lab-prepared 1N hydrochloric acid (49 ml conc. H_2SO_4 + 951 ml water). Use to keep all dispensers and bottles scrupulously clean. Store at room temperature.

4. Quality Control Material

a. Ausab EIA kit provides one vial of negative control and one vial of positive control. Swirl gently before use.

b. Three negative controls and two positive controls should be assayed within each analytical run.

c. If the difference between the positive and negative controls is less than 0.300 absorbance units, either the OPD reagents or the Ausab EIA kit reagents have deteriorated, and a new kit should be used.

5. Procedure

a. Remove OPD tablets from refrigerator to warm to room temperature. (Leave remainder of kit at 2-8 °C.)

b. Turn on water bath (40±1 °C).

c. Label reaction wells.
Position 1 = blank
Positions 2, 3, 4 = negative
Positions 5, 6 = positive
Positions 7, etc. = participant samples, run in duplicate

d. Pipet 200 ul of the appropriate control or participant specimen into its respective well.

e. Add one bead to all control and participant wells. To the blank well, add a pink filler bead. Also add filler beads to any incomplete rows.

f. Gently tap the side of the tray to remove trapped air. Beads must be immersed in the liquid. DO NOT SPLASH!

g. Cover the tray with paper seal. Label the tray with test name, date, and incubation finish time.

h. Float the tray in water bath (40±1 °C) for 2 hours ±5 minutes.

i. Shortly before the 2 hours are over, make up the conjugate reagent:
Volume B-HBs Ag required: (number of wells) X (0.11 ml)
Volume A-HPRO required: (number of wells) X (0.11 ml)
Mix well. Top bottle with 200 ul dispenser and prime CAREFULLY back into bottle. (Do not waste any!)
Place in position 1 on Pro-Quantum.

j. Remove tray from the water bath. Wash each well with 15 ml distilled water.

(1) Place tray in a riser rack on the washing track.
(2) Enter: the number of wells to be processed (1-999, divisible by 5, e.g., 50)
(3) Press WASH
(4) Enter: milliliters of water (15)
(5) Press GO
(6) After gears start to turn, GENTLY push the rack to connect with the gears.

k. Add 200 ul of conjugate to each well, except blank.

(1) Position the riser rack on the dispensing track.
(2) Enter: the number of wells to be skipped
(3) Press: SKIP
(4) Enter: the number of wells to be processed (exact number, e.g., 48)
(5) Press: DISPENSE
(6) Enter: Station number (1)
(7) Press: GO

l. Cover the tray with a paper seal. Label with test name, date, and incubation finish time.

m. Float the tray in water bath (40±1 °C) for 2 hours ±5 minutes.

n. Five to ten minutes before end of incubation, make up OPD reagent (reconstituted OPD must be used within 60 minutes).

(1) Add required number of OPD tablets to labeled OPD brown bottle, according to the number of wells used

No. of Wells	No. of Tablets	Diluent Volume
13	1	5 ml
28	2	10 ml
43	3	15 ml, etc.

(2) Add designated volume of diluent to the bottle. Let the bottle sit until the tablets dissolve.
(3) Mix well. Let the bottle sit to get rid of bubbles.
(4) Mix gently before applying 300-ul dispenser to ensure homogenicity.

(5) Apply the dispenser top.
(6) Immediately before dispensing, prime five times into waste and then prime back into bottle.

o. Remove the tray from the water bath. Wash each well with 15 ml distilled water as in step j.

p. Immediately invert the tray into the box of tubes. Match the A-1 position on the tray with the A-1 position in the box. Tap to drop all beads into the tubes. Remove pink bead from blank tube.

q. Place OPD reagent in position 2 of the Pro-Quantum.

r. Place the box on the rack and place the rack on the dispensing track.

s. Add 300 ul of OPD to each tube as in step k, except also add OPD to the blank. (Use station 2).

t. Cover and incubate for 30±2 minutes at room temperature (18-30 ^{o}C).

u. Place the 1N sulfuric acid bottle with the 1-ml dispenser in position 3 on the Pro-Quantum.

v. After 30 minutes, place the box of tubes on a rack on the dispenser track and add the sulfuric acid to each tube as in step k. (Use station 3.)

w. Agitate the tubes and remove the air bubbles.

x. On the Quantumatic, read program 9 within 2 hours after adding sulfuric acid. (See section on using the Quantumatic.) Read blank containing only OPD and sulfuric acid.

7. Results

a. The negative control value should be between -0.006 and 0.100 absorbance units.

b. The positive control value should be 0.300 absorbance units, or greater, after the negative control is subtracted from the positive control.

c. The cutoff value is obtained by adding 0.030 to the negative control mean absorbance value.

d. Results are considered reactive if the absorbance is greater than or equal to the cutoff value. Results are considered nonreactive if the absorbance is less than the cutoff value. In borderline reactive or borderline nonreactive results, the

absorbance value is within ±10% of the cutoff value, as long as the two readings agree. If the two readings on borderline values disagree, the sample should be retested.

8. References

a. Abbott Laboratories Diagnostic Division. Hepatitis B surface antigen (human) (subtypes ad and ay) Ausab EIA, enzyme immunoassay for the detection of antibody to hepatitis B surface antigen. North Chicago, Illinois: Abbott Laboratories Diagnostic Division, 1983.

b. Abbott Laboratories Diagnostic Division. Pro-Quantum bead washer and reagent dispenser operator's manual. North Chicago, Illinois: Abbott Laboratories Diagnostic Division, 1984.

V. ANALYTICAL METHODS

Z. Semen Analysis

1. Principle

Sperm measurements are obtained by using the Cellsoft automated semen analyzer (CASA). CASA, based on digitized image analysis, can recognize most sperm cells (except two-headed sperm or those with tail defects) and distinguish them from other semen constituents on the basis of their size, luminosity, and motion. These three factors constitute an integral component of the system's ability to provide accurate and objective data.

Sperm concentration and motility (including the percentage of motile cells, velocity, linearity, and lateral head displacement) are measured by using videotaped recordings of semen. For motility analysis, Cellsoft parameters are set at the beginning of the analysis phase and remain unchanged throughout the study. (These settings are given below.)

Morphology and morphometry (area, perimeter, length/width ratio, major axis length) are measured from sperm preparations on microslides. For morphology analysis, Cellsoft requires that the user "train" the software to recognize different sperm morphology classifications. During this process, Cellsoft is "taught" to recognize the size and shape of the sperm head and, the basis of a given set of criteria, classifies each sperm head as belonging to one of the five morphologic groups (normal, large, small, tapered, and amorphous). The only subjective contribution of the technologist is in setting the "threshold gray level" at the beginning of each analysis. This level is set VISUALLY. Day-to-day variations within and among technicians can be minimized by using a standard user training protocol. However, it is important to recognize this technician variability as a potential source of systematic error.

2. Cellsoft Equipment

a. One IBM PC/AT with Epson dot matrix printer
b. Two high-resolution black and white video monitors
c. One high-resolution video camera with microscope mount
d. Olympus phase contrast microscope and X10 objective
e. One VHS video cassette recorded (Panasonic)

3. Cellsoft Parameter Settings

a. Settings for sperm motility measurements:

Number of frames to analyze	15	
Number of frames per second	30	
Video standard (A/E)	A	
Minimum sampling--Motile	1	
--Velocity	4	
Maximum velocity (um/sec)	140	
Threshold velocity (um/sec)	10	
Cell color	White	
(Occular magnification)	(1.0)	(1.5)
Pixel scale (um/pixel)	0.688	0.459
Dilution factor	1.000	1.000
Cell size range (pixels)	5-25	11-56
Lateral head displacement:		
Minimum number of points	7	
Minimum velocity (um/sec)	18	
Minimum linearity	3.50	

b. Settings for sperm morphology classification

Cell color (choose one):		[X] black	[ ] white
Threshold grey level	--Low:	[80]	
	--High:	[114]	
Cell size range (f)	--Minimum	[3.000]	
	--Maximum	[40.000]	
Pixel scale (f/pixel):		[0.229]	
Default profile:		[CPILOT]	
Window coordinates:	--Top:	[130]	
	--Left:	[130]	
	--Bottom:	[365]	
	--Right:	[365]	

4. Specimen

The specimen is produced by masturbation and collected in a plastic all-purpose container with a screwcap lid. It is important to collect the entire amount. The participant is instructed not to use lubricants or condoms during collection. The specimen container must be protected from temperature change by placing it in a covered Styrofoam cup, and it should be taken to the laboratory within 30 minutes.

The following information is recorded on the label:

a. Date of collection
b. Time of collection
c. Days of abstinence
d. Spillage
e. Vasectomy status

5. Reagents and Supplies

a. 100% Alcohol (95% ETOH + 5% isopropyl), Baker
b. 95% Alcohol (95 ml of 100% alcohol + 5 ml H_2O)
c. 80% Alcohol (80 ml of 100% alcohol + 20 ml H_2O)
d. OG6, Harleco
e. Ea 50, Harleco
f. Histoclear, catalog no. HS-200, National Diagnostics
g. Histomount, catalog no. HS-103, National Diagnostics
h. Hematoxylin, Mayer's, Harleco
i. pH meter, Orion 701A
j. pH strips, ColorpHast, Em reagents, catalog no. 9583-9[illegible], range 6.5-10.0
k. Formalin solution (0.1%), 2 g neutral formalin, 10 g $NaHCO_3$, QS to 200 ml with distilled water
l. Incubation oven (30 °C)
m. Vortex
n. Slide staining rack
o. Microscope slides (frosted end)
p. Pasteur pipets (5-3/4 inches)
q. 5-cc syringes
r. 19-gauge syringe needles
s. Buffer, pH 7
t. Buffer, pH 10

6. Quality Control Material

a. Motility:

For quality control, a videotape of control specimens containing a low normal, a median normal, and a high normal sperm count is prepared and analyzed on the CASA once a day.

b. Morphology:

(1) Twenty slides made from one semen specimen from a healthy volunteer semen are used as a control. All 20 slides are analyzed initially, and 1 is used per day to verify the control.

(2) Ten slides prepared from 10 participant samples are analyzed one time in each analysis session as a "blind" control. The supervisor will randomly place these control slides into a daily run in a manner that makes them indistinguishable from the participant slides. These two control slides will be used throughout the study.

7. Procedure

a. Initial start-up:

(1) Allow 5 minutes warm-up for instrumental system and attachments.

Turn the power on in this order:

Microscope
Stage incubator (37 °C)
VCR
Video timer (may be left on so time and date do not have to be reset)
Camera

(2) Assure that the specimen incubator is at 30 °C. Adjust it if necessary. Record the temperature.
(3) Unlock the chute at the proper time.

b. Information documentation:

Record the following information on the worksheet.

(1) Participant identification number
(2) Agent Orange Projects Name
(3) Legal name
(4) Days of abstinence
(5) Collection time
(6) Vasectomy status
(7) Time of arrival in laboratory

c. Gross seminal evaluation:

(1) Liquefaction rate: By visually inspecting the specimen, determine whether liquefaction is complete or incomplete and report the time since ejaculation in minutes. (Allow the specimen to sit no longer then 2 hours before analysis.)

(2) Viscosity: One-half hour after collection, draw the semen into a transfer pipette and then slowly expel. Grade as follows.

Normal = discrete droplets

Minor
Moderate = increased thickness of the fluid,
Severe rate accordingly.

(3) Volume: The entire semen sample is drawn into a disposable 5-ml plastic syringe fitted with an 18- or 19-gauge needle. Record the volume to the nearest 0.1 cc. Deposit the sample back into the container.

(4) Color: Look at the color of the sample. Grade as follows:

Normal = gray-white, translucent

Brownish
Reddish = all abnormal
Yellowish
White

(5) Odor: DO NOT PURPOSELY SMELL THE SPECIMEN! If a specimen is not normal (musky), it will be obvious when the lid is removed. YOU MUST NOT INHALE ANY OF THE AEROSOLS. Report the smell as normal or abnormal.

(6) pH: The pH is determined directly in the collection container by using the Orion pH meter model 701A. (The pH meter is calibrated each day of analysis and a record of calibrations is maintained with the meter.) Measure to the nearest 0.1 pH unit. If the pH meter cannot be used because the specimen is too small, use pH strips.

d. Videotaping of specimen for concentration and motility:

(1) Mix the semen sample using the vortex for 15 seconds (with the lid on). Allow the sample to sit for a few minutes to dispel bubbles. Swirl before sampling.

(2) Using a Hamilton syringe, apply 5 ul of specimen to the center of the lower disc of the Makler chamber. Swirl syringe barrel in the specimen first, then aspirate from the middle of the specimen.

(3) Place the cover glass on the four tips. Check for an even spread without flooding. Avoid further touching of the cover glass.

(4) Lift the chamber by its handles and place it on the stage of the microscope.

(5) Focus on the grid, using the X10 phase objective, with the upper magnification at 1.0 and the lower at 10. (DO NOT use the X20 objective.)

(6) Looking at the color monitor, check to see that the sperm are shining whitely and that the background is comparatively dark. In the normal settings, the light-intensity diaphram is open all the way (about 8 or 9), and the condenser is all the way up. Check also to see that the digital time display is small and at the edge of the screen. If any part is in the taping area, it will interfere with the count!

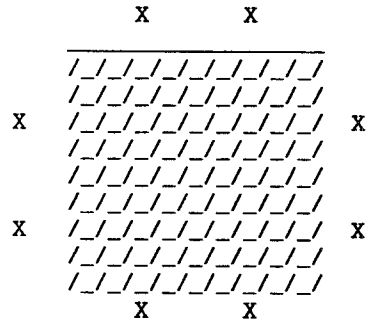
(7) Estimate the count by counting 10 squares. This number represents the concentration of sperm in millions per milliliter. Note the estimate on the worksheet.

(8) If the count is severely decreased, count the sperm in the entire grid area. Divide the number counted by 10 to get the concentration in millions per milliliter.

(9) Focus on an area outside but near the grid. (Allow no grid markings to show on the monitor.)

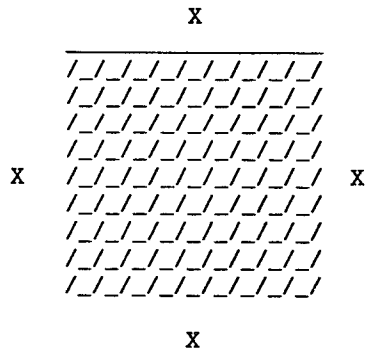
(10) Insert a "primed" tape (see Notes on Procedure) into the VCR. Press PLAY and RECORD simultaneously and note the starting time on the worksheet. Every 30 seconds, change fields. (Tape 10 squares first for 5-10 seconds each square to further verify the count made by Cellsoft. An estimated count may be made by using this taping before Cellsoft analysis.)

(11) For a normal count, record eight fields.



as marked by the Xs.

(12) For samples from subjects indicating vasectomies, record 4 fields for only 10 seconds each.



(13) When finished counting record a small bit of "blanked" fields to separate the specimens.

(14) Repeat steps 1-13 for each specimen. Be careful to note the start time for each.

(15) When finished, rewind the tapes remove, and label them with month, date, and letter denoting order.

i.e., / 0421 A /

Where 04 = April
21 = The twenty-first day.
A = First tape of the day.

Put this number on the worksheet beside each appropriate participant identification number.

(16) Store tapes in a cool, dry place until the analysis.

(17) Turn off the power in this order:

Camera
Stage incubator
VCR
Video timer (does not have to be turned off)

e. Preparation of slides for morphology:

(1) Make six slides:
(a) Four fixed in 95% ethanol.
(b) Two air-dried.

(2) Mix the specimen well.

(3) Label microscope slides with participant identification number.

(4) Place a large drop of seminal fluid on a slide.

(5) Place another slide on top of the drop. Press together and pull in the opposite direction.

Place each of the two resulting slides immediately into a Coplin jar containing 95% ethanol and let them remain there for 5 minutes.

(6) Repeat, for a total of four slides to be fixed. Then, for the two slides to be air-dryed, repeat step 5 and place the slides on the drying rack instead of in the ethanol.

(7) Remove the four slides from the ethanol and place them fluid side up on a drying rack.

(8) Place all six slides in a slide box for transport.

f. Concentration and motility analysis

(1) Turn on the VCR, the timer and both monitors (one for the actual image, one for the digitized image).

(2) Advance the tape to the time noted on the worksheet for the first participant.

(3) On the CRT at the C:/TF> prompt, enter CS (Cellsoft).

(4) Enter the accession number of the first participant sample to be analyzed. Check to see that the participant name is correct.

(5) Processing Samples

(a) At main menu type 5 (process samples).
(b) At next option type V to enter the volume.
(c) Enter the volume to the nearest tenth.
(d) At next option type R to calibrate the threshold.
(e) Using the > and < keys, adjust the size of the sperm images on the digitalizer until they are approximately the same size as the sperm on the live monitor and can be tracked across the screen without disappearing. Try to eliminate halos around the sperm. Note the threshold number.
(f) When the size is satisfactory, type Q for quit to return to the option screen.
(g) Type P to process the sample. The screen will state SET UP FIELD-HIT ANY KEY TO START PROCESSING.
(h) Rewind the tape to the START TIME for that participant, hit STOP, then PLAY.
(i) Hit any key on the keyboard to start processing. (Make sure the monitor image is stable.)
(j) Count all eight fields by typing N when prompted. I may be typed to ignore any fields that were unsuitable for counting because the images were unstable. In this case reanalyze the same field.

Note: If the sperm count is high, it may take longer than 30 seconds to analyze the field. The tape changes fields every 30 seconds. Hit STOP on the VCR to halt the tape. When you are ready for the next field, hit PLAY to continue.

(k) After eight fields have been analyzed, type E at the option screen to end the analysis.
(l) At the next screen the data are to be filed in THREE SEPARATE FILES by hitting the keys designated below:
F = To record summary data in file.
R = To record summary data in Cellsoft data base.
C = To record individual cell data in file.

(m) Once the data have been filed, process the next participant sample by typing N.
(k) Repeat all steps in (4) and (5) for each sample.
(l) Rewind the tape and store in a cool, dry place.

h. Morphology and morphometry analysis:

(1) Fixed slides are stained with the Papanicolaou (Pap) stain.

(a) Mayer's hematoxylin	5 minutes
(b) Running tap water	5 minutes
(c) 80% Alcohol	8 dips*
(d) 95% Alcohol	8 dips
(e) OG-6	2 minutes
(f) 95% Alcohol	8 dips
(g) 95% Alcohol	8 dips
(h) 95% Alcohol	8 dips
(i) EA-50	2 minutes
(j) 95% Alcohol	8 dips
(k) 95% Alcohol	8 dips
(l) 95% Alcohol	8 dips
(m) 100% Alcohol	8 dips
(n) 100% Alcohol	8 dips
(o) 100% Alcohol	12 dips
(p) Histoclear	12 dips followed by 5 minutes

*one dip = 1 second

(2) Remove slides one at a time and place a cover slip on each slide using Permount.

(3) Differential:
(a) Set the microscope so that the upper magnification is at 1.5 and the lower at 10.
(b) Count 200 sperm (100 if the count is less than 10 million by using the morphologizer program in the automatic mode. (If the smear has increased background material, the manual mode may have to be used.)
(c) Enter by typing MPN at the prompt, i.e., C:/TF>MPN
(d) Use the accession number assigned in test order. Enter the participant's demographics.
Enter the name in this format:
AOP,___-xxxxxx.
(e) Focus on the first field, adjust the lighting for optimum measuring. Use the X20 objective.
(f) Set the threshold so that the size of the digitized sperm match the sperm size on the live image. Type Q to quit and follow the instructions on the screen to begin processing.
(g) The Cellsoft software parameters have been previously set to classify sperm head shape as:
Normal
Tapered

Small
Large
Amorphous

(h) Cellsoft also records measures of morphometry.

(i) When the measurements have been completed, the information MUST be printed. Touch P to print the summary data only and F to file summary and individual data.

8. References

a. Belsey MA, Eliasson R, Gallegos AJ, Moghissi KS, Paulsen CA, Prasad MRN. Laboratory manual for the examination of human semen and semen-cervical mucus interaction. Singapore: Press Concern, 1980; 7-32.

b. Cannon DC. Seminal fluid. In: Henry JB, ed. Clinical diagnosis and management by laboratory methods. Philadelphia: WB Saunders, 1984:516-9.

c. CRYO Resources. Laboratory networking database management system truncated version labnet, New York, New York: CRYO Resources, 1985. [Unedited programmer's version.]

d. CRYO Resources. Cellsoft user manual. Version 3.0, 1st ed. New York: CRYO Resources, 1986.

e. Schrader SM. Semen analysis. Cincinnati: National Institute for Occupational Safety and Health, 1983; SOP No EA-60(1).

f. Urry R, Seminal fluid. In: Kjeldsberg CR, Knight JA, eds. Body fluids, laboratory examination of amniotic, cerebrospinal, seminal, serous and synovial fluids: a textbook atlas. Chicago: American Society of Clinical Pathologists, 1986:117-27.

V. ANALYTICAL METHODS

AA. Alco-Sensor Breath Analyzer

1. Principle

The Alco-Sensor is a pocket-sized screening device designed to read breath alcohol concentrations. Only a disposable mouthpiece and a long-lived battery are necessary to keep the Alco-Sensor operational.

The accuracy of any breath alcohol test depends on the relationship between the concentration of alcohol in the blood and that in the deep lung breath. This ratio, which is 2,100 to 1, is well established.

The amount of alcohol in a properly collected breath sample is governed by the amount of alcohol in the blood in the lungs. To get an accurate blood alcohol reading, a deep lung breath sample must be collected and analyzed.

2. Equipment and Supplies

a. Alco-Sensor.

The Alco-Sensor is designed to operate at temperatures between 20-36 °C. Temperature is important because it affects the rate of the electrochemical reaction. This unit can operate at temperatures as low as 0 °C, but the response is sluggish and some accuracy is sacrificed. The optimum temperature for operation is 20-36 °C.

Instrument temperature can be checked by the liquid crystal thermometer built into the back panel. It reads the temperature in centigrade degrees and has a range of 20-36 °C. If no figure is visible, the unit is either too hot or too cold. A unit that is too cold can be placed in the shirt pocket to bring it to operating temperature in a short time.

Once the unit is at operating temperature, it will function properly in ambient temperatures of 0-100 °C, atmospheric pressure of 635-787 mm of mercury, and relative humidity of 0-100%.

The higher the operating temperature, the more efficient the unit becomes. These higher temperatures minimize condensation and assure rapid reading. The unit becomes reusable in a shorter period of time. Tests can be run every 2 minutes.

b. Disposable mouthpieces.

c. Nalco calibration standard.

Nalco characteristics

- Nalco calibrator cans give the same value consistently at any room temperture.
- Nalco calibrator cans give at least 20 tests.
- For refilling, Nalco calibrator cans in good condition can be returned to:

  Intoximeters, Inc.
  1901 Locust Street
  St. Louis, MO 63103

- Mini Nalco calibrator cans have a shelf life of 3 months. Thereafter, they may drop in value 0.01 every 2 months.

- Because Nalco is a gaseous mixture, shaking the can will not show whether the can is full or empty. If pressing the valve does not release gas, the can is empty or the black valve is faulty.

d. Use only an alkaline 9-volt battery.

3. Calibration

a. Initial calibration checks are run daily. To check calibration:

(1) Alco-sensor should be brought to temperature with the SET buttom depressed.

(2) Depress READ button and note at least a momentary flashing .000. If this number is not seen, depress SET button for a few moments and recheck.

(3) Attach mouthpiece and deliver standard solution.

(a) Make airtight connection with mouthpiece at short end of Alco-Sensor.

(b) After depressing SET button, press valve head down, permitting standard to flow through mouthpiece.

(c) After 3 seconds press READ button (to take sample) and then release valve head.

(4) Keep READ button depressed until reading reaches a maximum value. Reading should correspond to value of standard. (If not, proceed to Section b.) Wait 20 seconds. Depress READ button. While the button is held down, the liquid crystal display area should flash for a moment showing .000.

b. To recalibrate:

(1) Turn CONTROL B clockwise three turns.

(2) Repeat step a.(3)-(4).

(3) When the display reads higher than the true value of the standard, turn B counterclockwise until the correct value is displayed. Keep the READ button depressed and continue to adjust B counterclockwise if the number on the digital display continues to increase in value.

c. Verify calibration by rerunning steps a.(1)-(4).

4. Procedure

a. Remove unit from the box and note the liquid crystal reading on the back of the unit; a temperature of 20-36 ^{o}C indicates that the unit is in the operating range.

b. To check for contamination, attach a mouthpiece, press READ button, and hold down. Check to see if .000 is constant.

Caution: Sufficient time after each test must be allowed for all traces of alcohol on the cell surface to be eliminated. When the READ button is depressed all the way down, the digital display should show .000 with the "-g" flashing at least once or twice when the READ button is held down for 10 seconds. If this does not occur, depress SET button for a few minutes. This action will short circuit the cell and accelerate the destruction of any alcohol left on the cell. Recheck to see that display shows .000.

c. Depress the SET button to set the valve and prepare for sample taking.

d. Obtaining the sample.

(1) Instruct the subject to give a sample. Ask the subject to blow continuously for as long as possible. It takes about 3-5 seconds to empty the lungs through the mouthpiece. (NO SMOKING is permitted within 3 minutes of the test because smoke will permanently damage the fuel cell.)

(2) Before the subject stops exhaling, push the READ button,

e. Keep READ button depressed until the reading stabilizes. Read maximum reading attained and record on work list or log sheet.

f. Push SET button to purge the cell and prepare for the next sample.

5. References

a. Intoximeters. Manual for Alco-Sensor. St. Louis: Intoximeters, 1984.

V. ANALYTICAL METHODS

BB. Melioidosis
Antibodies to Pseudomonas pseudomallei in Serum

1. Principle

A microhemagglutination procedure is used to test for melioidosis.

Inactivated serum is mixed, in serial dilutions, with sheep red blood cells (SRBCs) sensitized to the antigen for strains of Pseudomonas pseudomallei. A participant who has been exposed to any of the various strains of Pseudomonas has an antibody in the serum, which reacts with the sensitized SRBSs. The degree of sensitivity depends on the participant's present titer.

2. Specimen

Freshly drawn serum is used. Fasting is not required.

For testing not done immediately, freeze serum at -10 °C or lower.

Minimum sample size: measurable as 0.05 ml.

Optimum aliquot: 0.5 ml.

3. Reagents, Supplies, and Storage

a. Saline (0.85%)
Weigh 42.5 g NaCl. QS to 5 L with distilled water.

Store at room temperature (22-28 °C). The solution is stable indefinitely.

b. Stock PBS (0.25 M phosphate buffered saline).
Weigh 27.4 g Na_2HPO_4 (anhydrous)
7.88 g $NaH_2PO_4 \cdot H_2O$
Q.S. to 1 L with distilled water.
Store at room temperature (22-28 °C).
This solution is stable for 12 months.

c. Working PBS (0.01M phosphate buffered saline)
Measure 40 ml stock PBS (0.25M)
Weigh 8.5 g NaCl
Q.S. to 1 L with distilled water.
Store at room temperature.
The solution is stable for 3 months.

d. Stock BSA (0.6% bovine serum albumin)
Measure 3 g BSA
QS to 500 ml with working PBS (0.01M)
Freeze in 50-ml aliquots at -10 °C or lower.
The solution is stable indefinitely.