Bulletin

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Measuring Bilirubin on Whole Blood

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1 Introduction

Traditionally bilirubin measurements are always taken on plasma or serum samples. This involves a certain amount of sample preparation prior to the measurement.

With the addition of versions ABL™735 and ABL730 to RADIOMETER™'s ABL700 Series of blood gas analyzers, it is now possible to quantitatively measure bilirubin on whole blood samples. The ABL735 and ABL730 can measure bilirubin together with the oximetry parameters on as little as 35 µL of whole blood. This means that bilirubin is available as a STAT parameter and the time-consuming sample preparations are no longer necessary.

2 Summary

This bulletin describes the measuring principles behind the optical measurements of bilirubin on whole blood, showing how the absorbance spectrum of whole blood is affected by the presence of bilirubin.

The reference method used for bilirubin measurements and the performance and interference tests run in connection with the measuring of bilirubin are also described.

3 Measuring Principles

3.1 Bilirubin

Bilirubin is a family of orange-yellow pigments produced from the breakdown of the heme group in the red blood cells. An increased concentration of bilirubin in the blood (hyperbilirubinemia) leads to a yellow discoloring of the skin and is the cause of jaundice/icterus in newborns. Hyperbilirubinemia is also seen in patients with liver failure and hemolytic diseases such as thalassemia.

3.2 Total Bilirubin Concentration (*c*tBil)

The ABL735/730 measures the total bilirubin concentration, i.e., the sum of the concentration of the two major types of bilirubin found in blood; unconjugated (indirect) and conjugated (direct). Unconjugated bilirubin takes up by far the largest part of the bilirubin present in neonatal blood.

The two types have slightly varying spectra; the ABL reports the total bilirubin amount.

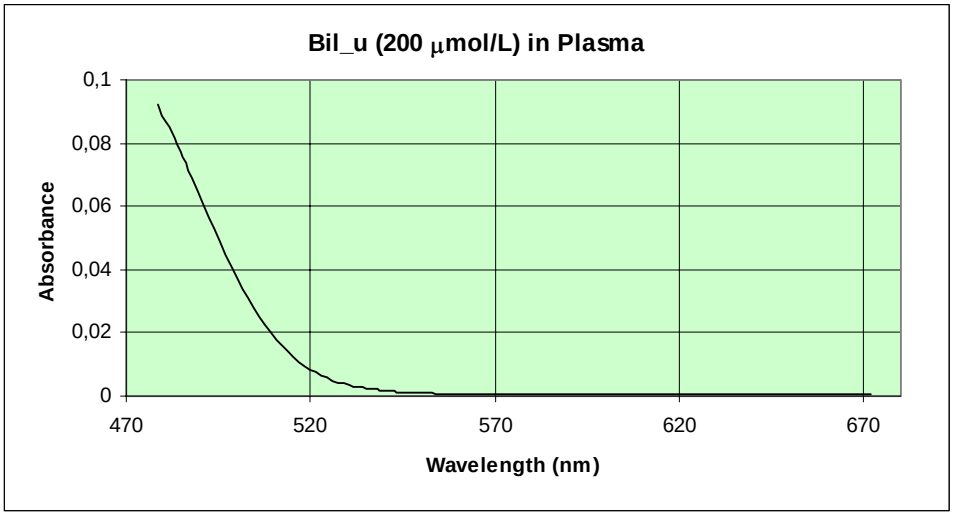
In neonates it applies that [1]:

Total Bilirubin	=	Unconjugated Bilirubin	+	Conjugated Bilirubin
*c*tBil	=	*c*Bil_u	+	*c*Bil_c

3.3 Bilirubin Spectrum

Bilirubin displays an absorbance spectrum within the wavelength range of the spectrometer in the oximetry module of ABL700 Series of analyzers (478 - 672 nm). As the spectrum of bilirubin can be distinguished from the rest of the blood spectrum, it is possible for the ABL700 Series analyzer to determine how much is present.

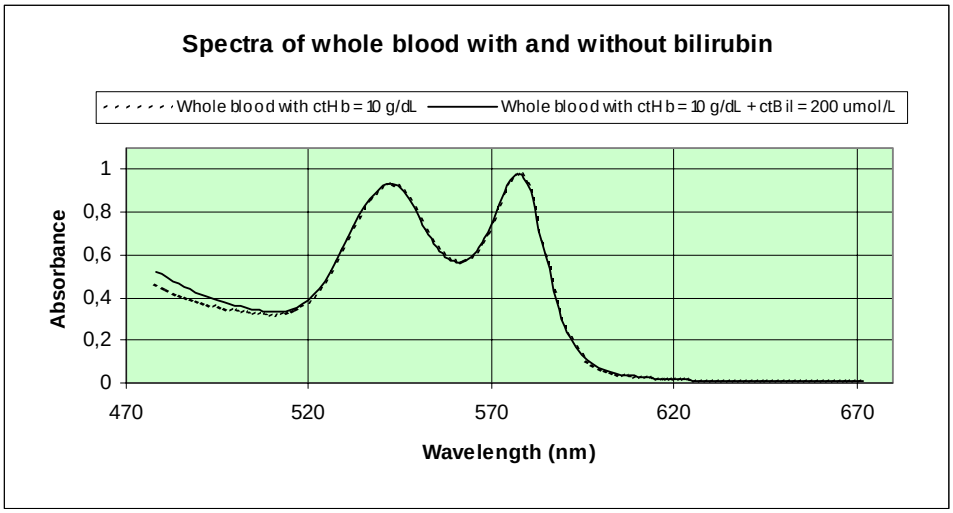
The spectrum of unconjugated bilirubin at a concentration of 200 µmol/L is depicted below.



The basic principles behind spectrometry measurements on the ABL700 Series can be found in Bulletin No. 2-1998, The ABL™700 Oximeter: A Technical Overview, March 1998 [2].

3.4 Bilirubin in Whole Blood

The diagram below shows two absorption spectra of fully oxygenated, hemolyzed whole blood, one spiked with 200 µmol/L unconjugated bilirubin and the other unspiked.



It is the difference in the two spectra, seen between about 478 and 500 nm, which enables us to measure the bilirubin content in whole blood samples.

3.5 ctBil(P) vs. ctBil(B)

Bilirubin is found only in the plasma phase of blood, hence it is the bilirubin concentration in plasma that is reported.

In order to measure the hemoglobin and dyshemoglobin content of blood spectroscopically, the ABL700 Series analyzers hemolyze the red blood cells. The hemolyzation process is performed in the cuvette by ultrasonic vibration and destroys the cell wall of the red blood cells, thereby releasing the hemoglobins. Once the hemoglobins are released, light scattering from the cell walls is removed, and an accurate absorption spectrum can be recorded.

However, on hemolyzation of the red blood cells, it is not only the hemoglobins that are released into the plasma. The red blood cells contain a certain amount of liquid, intracellular fluid. This fluid is also released on hemolyzation, and has the effect of diluting the bilirubin concentration in the plasma.

In this way the *c*tBil(P) is higher than the directly measured *c*tBil(B) and the difference must be corrected for.

3.6 Correcting for the difference in *c*tBil(B) and *c*tBil(P)

The following equation shows how the ABL corrects for dilution of *c*tBil on hemolysis:

$$c\text{tBil}_{\text{plasma}} = \frac{c\text{tBil}_{\text{blood}}}{1 - \text{Hct}_{\text{calc.}}}$$

where:

*c*tBil(P)	=	concentration of total bilirubin in the plasma phase
*c*tBil(B)	=	concentration of diluted total bilirubin after hemolyzation (directly measured)
$\text{Hct}_{\text{calc.}}$	=	calculated hematocrit (a fraction)
	=	0.0301 x *c*tHb (in g/dL) - *see below for further explanation*

3.7 Hematocrit

The correction for dilution of plasma bilirubin on hemolysis involves the hematocrit value. Hematocrit is a fraction (or percentage) describing the volume the red blood cells occupy in a whole blood sample.

$$\text{Hct} = \frac{\text{volume of red blood cells}}{\text{volume of whole blood}}$$

The hematocrit value can be measured, as for example in the ABL555 and the ABL70 analyzers (also from RADIOMETER), however in the ABL700 Series analyzers it is calculated from the value for the total hemoglobin concentration.

$\text{Hct}_{\text{calc.}}$ = 0.0301 x *c*tHb, where *c*tHb is in g/dL[1]

The constant, 0.0301, is calculated from using an expression for the average concentration of hemoglobin *within* the red blood cells. This expression is known as the mean corpuscular hemoglobin concentration, MCHC, and is explained further below.

3.8 MCHC

As mentioned above, mean corpuscular hemoglobin concentration, or MCHC, expresses the average concentration of hemoglobin *within* the red blood cells. The MCHC is used to determine Hct according to the following formula:

$$\text{Hct} = \frac{c\text{tHb}}{\text{MCHC}}$$

The MCHC can vary from patient to patient and is dependent on amongst other things, the iron (hemoglobin) content of the blood. The reference range for newborns is 320 - 350 g/L [3].

The ABL735/730 uses a standard MCHC value of 332 g/L (33.2 g/dL).

1) To convert *c*tHb in mmol/L to g/dL multiply by 1.6114

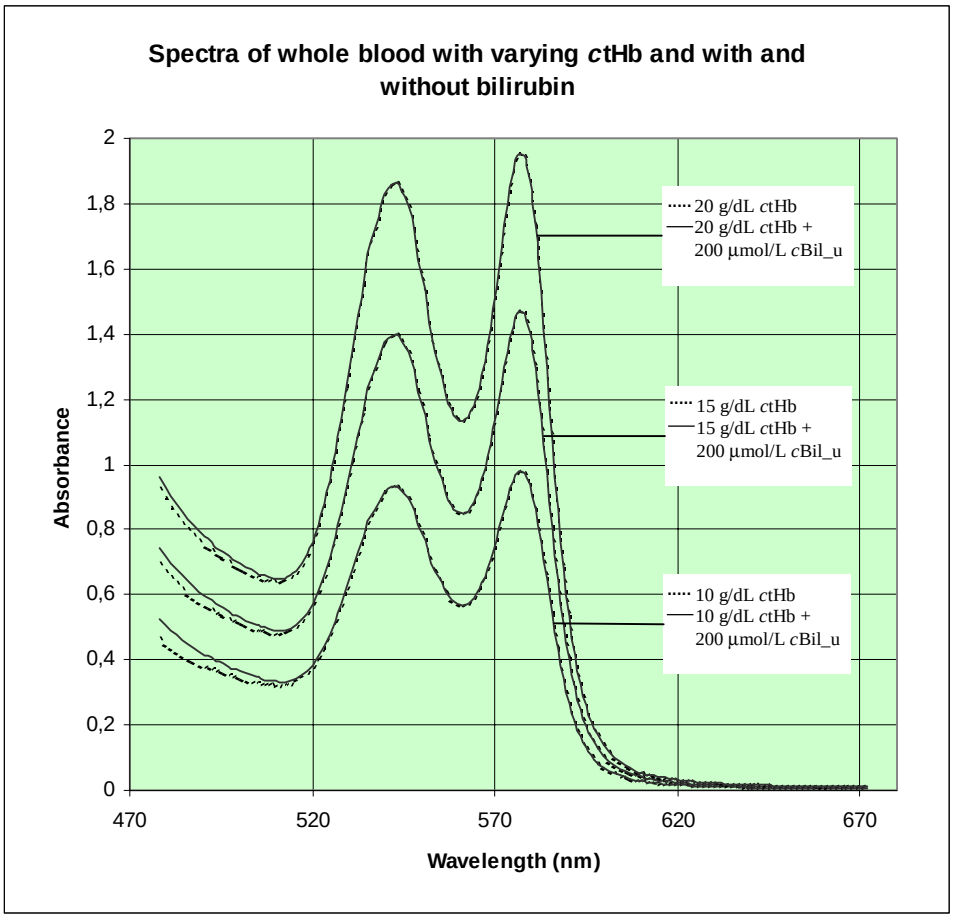
Considerable deviations from the standard MCHC value will influence the $Hct_{calc.}$ and ultimately affect the *c*tBil measurement. It is possible to correct the displayed *c*tBil in these incidences if the true Hct value is known [4].

3.9 Conditions of Measurement

There is a condition for the reporting of *c*tBil in that *c*tBil will not be reported by the analyzer if *c*tHb > 25 g/dL (15.5 mmol/L). At *c*tHb levels above 25 g/dL the calculation of Hct is no longer reliable enough to use in the calculation of *c*tBil(P). This is because at high *c*tHb levels, $Hct_{calc.}$ approaches 1 and the closer $Hct_{calc.}$ is to 1, the greater the effect of (1 - $Hct_{calc.}$) in the equation for *c*tBil(P) and therefore, the more marked the errors in Hct calculation. This will then be reflected in determinations of *c*tBil(P).

Also at high *c*tHb, dilution of *c*tBil on hemolyzation will be greater, thus reducing *c*tBil(B) and giving a smaller spectroscopic signal.

The diagram below shows the effect of tHb levels on the spectra of fully oxygenated, hemolyzed whole blood, unspiked and spiked with bilirubin.



It can been seen in the above diagram that the higher the tHb level is, the less the difference in the spectra when bilirubin is added, and thereby the greater the measurement uncertainty. This illustrates the constraints described above.

3.9 Sample Volumes

The ABL700 Series is able to measure a variety of different parameter profiles on samples down to 35 µL. For example the automated micromodes available on the ABL735/730 are:

Sample Volume	Parameters
35 µL	Oximetry/*F*HbF/*c*tBil
35 µL	Glucose/lactate (ABL735 only)
55 µL	pH/blood gases/oximetry/*F*HbF/*c*tBil
95 µL	pH/blood gases/oximetry/electrolytes/glucose+lactate (ABL735 only)/*F*HbF/*c*tBil

4 Reference Methods

4.1 Definition

A reference method is an established analytical process for the determination of concentrations of specified substances.

4.2 NIST SRM 916a

NIST (National Institute of Standards and Technology) Standard Reference Material 916a is unconjugated bilirubin in pure form. It is this standard material which all bilirubin measuring methods should be traceable to.

NIST SRM 916a is used to validate and verify the NCCLS (National Committee for Clinical Laboratory Standards) recommended reference method, the Jendrassik-Gróf method modified by Doumas [5,6].

The NIST SRM 916a material was used by RADIOMETER as a working standard in measurements of the ABL735/730 against the chosen reference method.

4.3 Reference Method for *c*tBil on ABL735/730

The reference method used by RADIOMETER for measuring *c*tBil involved using the NIST SRM 916a material as a calibrator for a Hitachi 717 Wet Chemistry Analyzer situated at Medi-Lab, Copenhagen [7].

The process of this calibration and the following measurements are outlined below.

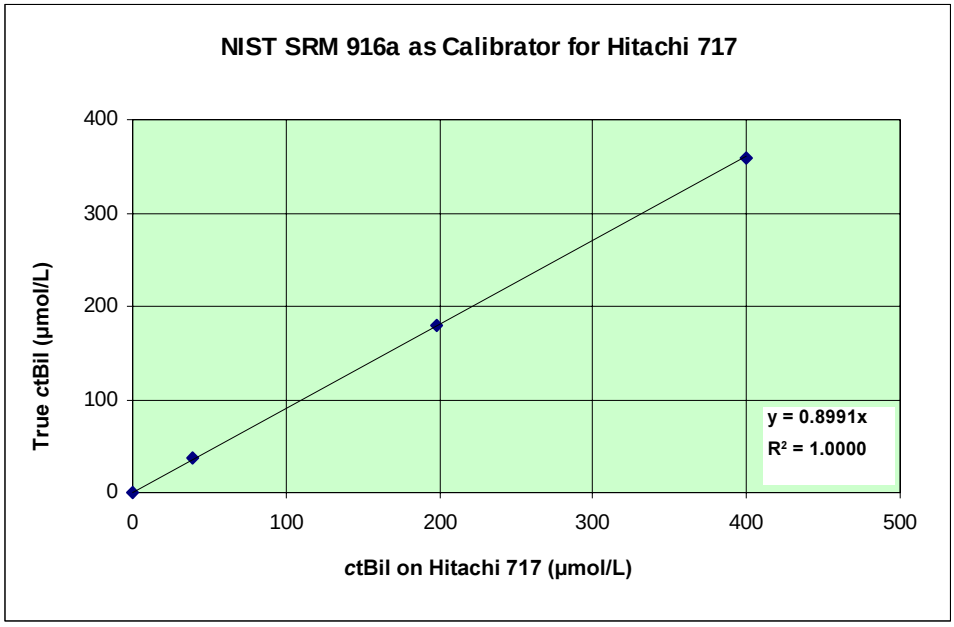
4.4 NIST SRM 916a as a Calibrator of Measurements on the Hitachi 717

The following table describes the process by which NIST SRM 916a was used as a calibrator of bilirubin measurements on the Hitachi 717 at Medi-Lab.

Step	Action
1.	A bulk bilirubin solution (unconjugated) was prepared according to the NIST Certificate of Analysis for SRM 916a. The procedure is also described in detail in [5].
2.	This bulk solution was then used to prepare bilirubin solutions in the following concentrations: • 0 µmol/L • 34 µmol/L • 170 µmol/L • 340 µmol/L

Step Action, cont'd

3. The bilirubin concentration of each solution was measured on the Hitachi 717 at Medi-Lab. The results of these measurements were then plotted against the true concentrations. An example plot is shown here:

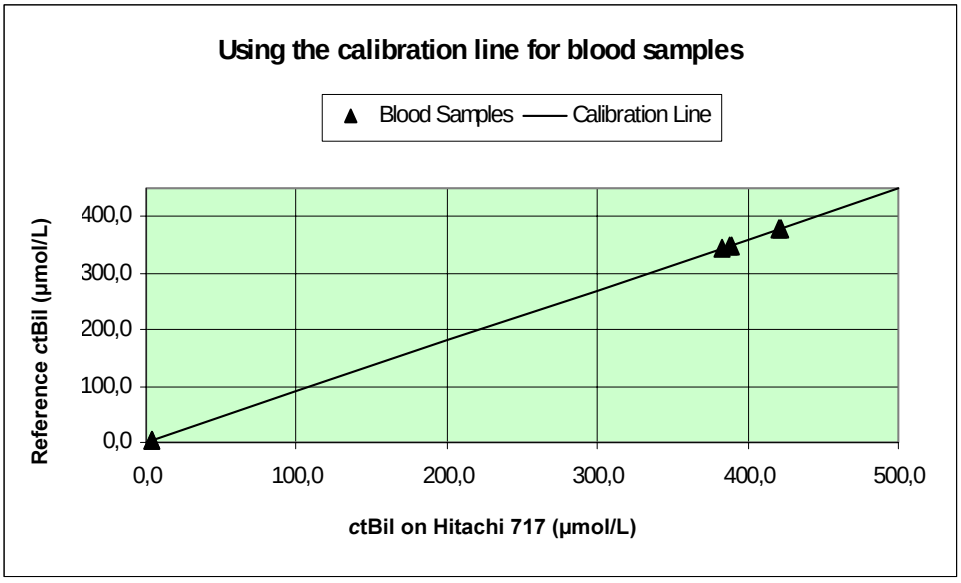


4. This plot was then used as the calibration line for the measurements on blood samples (*see below*). A new calibration line was plotted with every batch of blood samples sent to Medi-Lab.

4.5 Using the Calibration Line

Fully oxygenated blood samples at different tHb levels were spiked with unconjugated and conjugated bilirubin and pH adjusted to about pH = 7.4. These samples were analyzed for *c*tBil on 5 ABL735 analyzers at RADIOMETER. The same samples were sent to Medi-Lab where *c*tBil was measured on the Hitachi 717.

Measurement results from the Hitachi were adjusted according to the calibration by NIST standard material (*described above*) and the adjusted values compared to those obtained from the ABL735. The diagram below is an example of some of the results obtained from this procedure:



The data for the blood samples is shown in the table below:

*c*tBil of blood sample as recorded by the Hitachi 717 (µmol/L)	Reference *c*tBil of blood sample as read off from the calibration line (µmol/L)
4.0	3.6
4.0	3.6
383.0	344.4
389.0	349.8
388.5	349.3
422.0	379.4
421.0	378.5

Reference values for the ABL735/730 obtained in this way were used to calibrate the bilirubin measuring process in the analyzer in such a way as to ensure minimal deviation of *c*tBil measurements on the ABL from the reference method.

4.6 Fine Tuning

Due to a slight difference in the spectra of 'synthetic' bilirubin that was used to spike the samples in the laboratory, and the 'natural' bilirubin that is produced by the body, the measuring process required further fine tuning.

The algorithms used for the measurement of bilirubin account for this spectral difference and have been calculated using results from studies at neonatal units.

5 Performance and Interference Tests

5.1 Performance

Having compared the ABL735/730 measurements with the reference method and made the appropriate calibrations to the measuring process, the performance of the analyzers has then to be established.

The performance of the ABL is established during specification tests (*described below*).

5.2 Specification Tests

To establish the performance of the ABL735/730, a whole series of specification tests were performed using combinations of the following parameters:

- 5 analyzers, all measuring modes.
- Adult plasma samples spiked with unconjugated and conjugated bilirubin.
- Adult blood samples spiked with bilirubin at concentrations of about 200 and 400 µmol/L at different tHb levels.
- Each sample measured 5 times on each analyzer.
- Measurements taken over at least 3 days.

5.3 Verification Tests

It is necessary to verify the specification tests in a series of verification tests. Verification tests are a repetition of the specification tests on 5 different analyzers.

5.4 Reporting of Performance Characteristics

In total about 9,000 measurements were taken during specification and verification tests to establish the performance characteristics of the ABL735/730 analyzer. The performance characteristics are expressed in a series of standard deviations and inaccuracies, all of which are reported in the ABL700 Series Reference Manual [8].

5.5 Interference Tests

The *c*tBil measurements on the ABL735/730 analyzers have also been tested for interfering substances, i.e. we have tested whether the *c*tBil measurements are influenced by other substances commonly found in blood. Interference tests are performed by comparing the *c*tBil from unspiked samples and samples that have been spiked with the interfering substance.

5.6 Interfering Substances

The substances tested for interference on bilirubin measurements are listed below. They were tested in normal concentrations, i.e. concentrations normally expected of the substance if present in the blood stream.

- Intralipid
- Betacarotene
- pH
- Cardio Green
- Evans Blue
- Sulfhemoglobin, SHb
- Fetal Hemoglobin, HbF
- Variations in MCHC
- *Cyanmethemoglobin, HiCN* [2]
- *Patent Blue V*
- *Methylene Blue*

Of the substances listed above, only those written in italics showed any significant interference on bilirubin measurements. Detailed results from the interference tests on ABL735/730 are presented in the ABL700 Series Reference Manual [8].

References

1 American Academy of Pediatrics. Provisional Committee for Quality Improvement and Subcommittee on Hyperbilirubinemia. Practice parameter: management of hyperbilirubinemia in the healthy term newborn. Pediatrics 1994; 94, 4: 558-65.

2 Krarup T. The ABL™700 Oximeter: a technical overview. Radiometer Publication Bulletin No. 2-1998. Copenhagen: Radiometer Medical A/S, 1998. Code No. 918-556.

3 Lentner C, ed. Geigy Scientific Tables. Basle: Ciba-Geigy Ltd.1984: 3.

4 The Optical System. In: ABL™700 Series Reference Manual. Ed. C. Copenhagen: Radiometer Medical A/S, 1999: Chapter 6, 1-16. Code No. 989-312.

5 Doumas BT, Perry BW, Bayse DD, et al. Candidate reference method for determination of bilirubin in serum: test for transferability. Clin Chem 1983; 29: 297-301.

6 Doumas BT, Kwok-Cheung PP, Perry BW, et al. Candidate reference method for determination of total bilirubin in serum: development and validation. Clin Chem 1985; 31: 1779-89

7 Medi-Lab, 5-7 Adelgade, P.O. Box 2, DK-1001 Copenhagen, Denmark

8 Performance Characteristics. In: ABL™700 Series Reference Manual. Ed. C. Copenhagen: Radiometer Medical A/S, 1999: Chapter 8, 13-22. Code No. 989-312.

2) The 'normal' level for HiCN is 0 mmol/L, the test level was 0.11 mmol/L

Subject	Title	Code No.
No. 1-1998:	Critical Limits	918-555
No. 2-1998:	The ABL™700 Oximeter: A Technical Overview	918-556
No. 3-1998:	The ABL™700 Oximeter: New Standards in Interference Detection	918-557
No. 4-1998:	RADIOMETER's Hemolyzing Cuvette	918-558
No. 5-1998:	The ABL™700 Series' Lift'n'Go™ Inlet	918-559
No. 6-1998:	Guideline to Measurement on the ABL555 Using the User-specified Fluid Mode	918-560
No. 7-1998:	ABL™70: Reliability in Point of Care Testing	918-561
No. 8-1998:	Glucose Measuring System - What Is Measured And What Is Reported?	918-562
No. 9-1998:	Year 2000	918-563
No. 10-1999:	Calibration of pH and pCO_2 Sensors on the ABL™70	918-577
No. 11-1999:	Acid-Base Chart - easy interpretation of the acid-base status on the ABL™700 Series	918-578
No. 12-2000:	The ABL™700 Series' Autocheck Module: An Innovative Piece of QC Technology	918-594
No. 13-2000:	Save Time in STAT Testing	918-592
No. 14-2000:	RADIANCE™ - Remote Control Functions for RADIOMETER™ ABL™ Analyzers	918-597
No. 15-2000:	Measuring Bilirubin on Whole Blood	918-596
No. 16-1999:	RADIANCE™ / ABL™700	918-602
No. 17-2000:	The RADIOMETER™ Transcutaneous pO_2 Electrode, E5250	918-616
No. 18-2000:	Discover QualityGuard™ NPT™7's Internal Quality Control System	918-618
No. 19-2000:	Preparation of Bilirubin Calibration Solutions	918-619
No. 20-2000:	NPT™7 Measuring Principles	918-620
No. 21-2001:	NPT™7 Clinical Laboratory Improvement Act (CLIA) Compliance	918-625
No. 22-2001:	Frequently Asked Questions Concerning NPT™7 Quality Control/Quality Assurance	918-626
No. 23-2001:	Hyperbilirubinemia and Requirements to the Determination of the Concentration of Bilirubin	918-627
No. 24-2001:	Guide to Evaluating Bilirubin Measurements - Designing the Optimal Test	918-628
No. 25-2001:	Comparison of the QC Methods of the ABL™700 Series	918-630
No. 27-2001:	Development of RADIOMETER™ Quality Control Products	918-640
No. 28-2001:	Production and Metrological Traceability of Quality Control Products	918-641