Non-esterified Fatty Acids (NEFA)

Fujifilm HR Series NEFA-HR 2 (Fujifilm Wako Code No. 999-34691)

6/18/2020 MR/WK; rev 1-7-2021 wk

*Read package insert (**Addendum Figure 1** automated method 2018 and **Addendum Figure 2** microplate method 2019) before running this analysis. If the 2018 package insert has been updated, this procedure must be re-evaluated. wk*

Specimen: Serum separated from cells as soon as possible. Store frozen at -20°C until analysis.

Icteric, hemolyzed and lipemic sample assay readings (interference from sample) are automatically corrected by subtracting non-reacted well absorbance (Read 1 at 550 nm) from reacted-well absorbance (Read 2 at 550 nm).

Reagents:

Color Reagent A [999-34691 HR Series NEFA-HR (2)]**:** Must be reconstituted. Contains: acyl-CoA synthetase, CoA, ATP, 4-aminoanitpyrine, ascorbate oxidase, and sodium azide. Store refrigerated at 4°C.

Solvent A [995-34791 HR Series NEFA-HR (2)]**:** Contains: phosphate buffer and sodium azide. Store refrigerated at 4°C.

Color Reagent B [991-34891 HR Series NEFA-HR (2)]**:** Must be reconstituted. Contains: acyl-CoA oxidase and peroxidase. Store refrigerated at 4°C.

Solvent B [993-35191 HR Series NEFA-HR (2)]**:** Contains: 3-methyl-N-ethyl-N-(β-hydroxyethyl)-aniline. Store refrigerated at 4°C.

NEFA Standard Solution (1 mmol/L = 1 mEquiv/L) [Fujifilm Wako #276-76491]**:** Supplied in liquid form and ready to use. Store refrigerated at 4°C.

18 MOhm water (18MΩ H_2O): from the water purification system in Lab 109/110.

Control. In-house pooled serum sample. Store frozen at -20°C. Thaw only the amount needed for the day; use a new aliquot each day. Control ranges (average ± 2SD) to be determined.

0.85% (w/v) Sodium chloride (for diluting high NEFA samples) [saline]: In a 500-mL volumetric flask, dissolve 4.25 g NaCl (MW = 58.44 g/mol) in ~ 400 mL 18MΩ H_2O. Mix. Bring up to 500 mL with 18MΩ H_2O. Mix. Store in a labeled container refrigerated at 4°C; stable 6 months.

Procedure:

1. Check that the Synergy H1 Microplate Reader (Biotek, Winooski, VT) in Hultz 135 is available for use. Sign up to use it on the Departmental Microplate Calendar. Turn on the computer that controls the plate reader. The password is “Nutrition135.”

2. Thaw serum SAMPLEs and CONTROL. Vortex gently to mix.

3. Remove **Color Reagents A and B, and Solvents A and B** from the refrigerator.

For **Working Color Reagent A**: Add one bottle of **Solvent A** to one vial of **Color Reagent A**. Mix gently by inverting the vial until contents are completely dissolved. *Reconstituted solution is stable for one month at 4°C.*

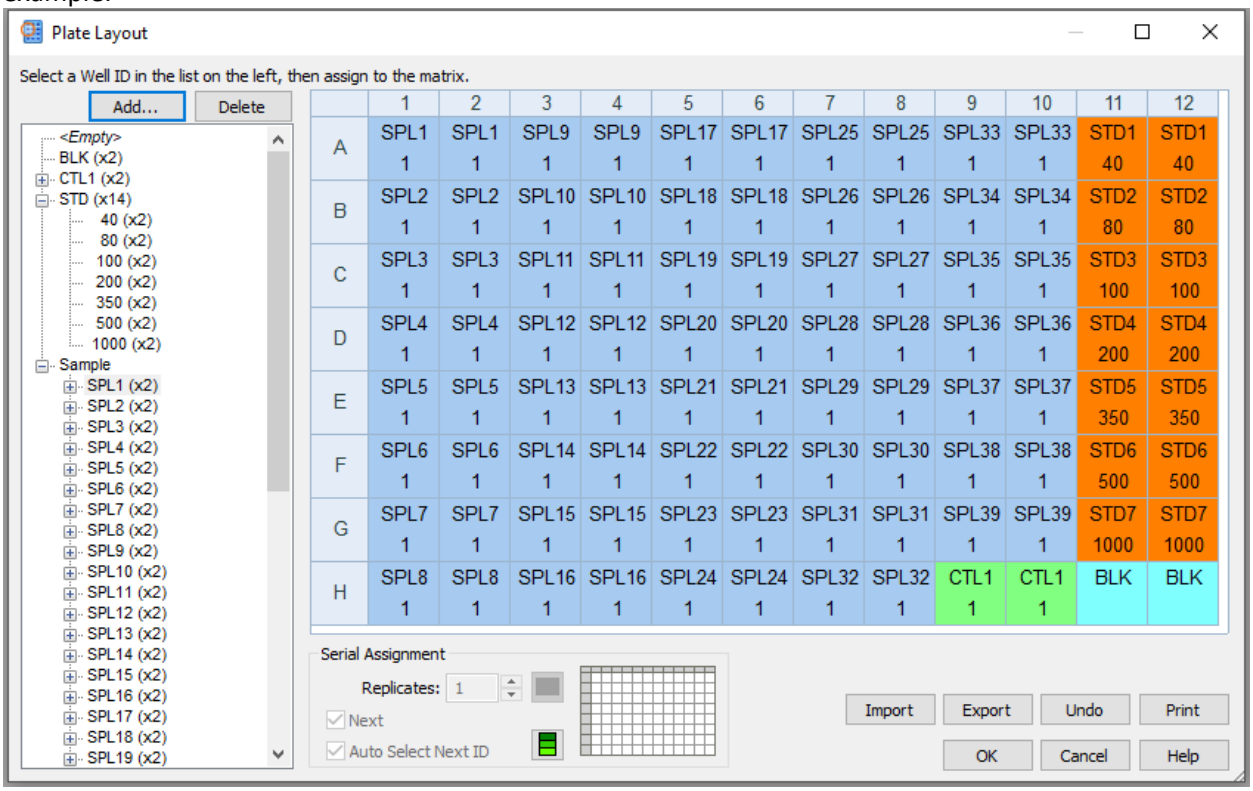
For **Working Color Reagent B**: Add one bottle of **Solvent B** to one vial of **Color Reagent B**. Mix gently by inverting the vial until contents are completely dissolved. *Reconstituted solution is stable for one month at 4°C.*

4. Prepare NEFA Working STANDARDs fresh, daily, in 1.5-mL microtubes following **Table 1** below.

Table 1. Working NEFA standard set preparation using a 1 mmol/L NEFA Standard for a total volume of 300 µL of each NEFA Working STANDARD.

Standard number	**Amount of 1 mmol/L NEFA Standard (µL)**	**18MΩ H_2O (µL)**	**Final NEFA Working STANDARD concentration (µmol/L)**
BLANK	0	1000	0
Std 1	12	288	40
Std 2	24	276	80
Std 3	30	270	100
Std 4	60	240	200
Std 5	105	195	350
Std 6	150	150	500
Std 7	300	0	1000

5. Plan the layout of a 96-well microtiter plate (96-well flat bottom, medium binding, polystyrene without lid; Greiner Bio-one 655101) according to the existing protocol. See **Figure 1** plate layout example.



6. Turn on the Synergy microplate reader (right, bottom, front side). Open Gen5 2.04 program located on the desktop. Allow the instrument to do start up functions. Door opens when complete. Manually close door. Preheat the microplate reader to 37°C.

[Task Manager] menu

[Experiments] menu

[Create using existing protocol]. To find protocol go to [Documents] folder; [Lab 135] folder; [Protocols] folder; [NEFA] folder; filename NEFA Fujifilm HR Series Protocol Lab 135.

Check the [Protocol] for correct [Procedure]: [Temperature: Setpoint 37°C; Shake linear for 15 s; Delay for 5 minutes; Read (A) 550; Plate Out,In Add Reagent B; Shake linear for 15 s; Delay for 5 minutes; Read (A) 550].

Check [Plate Layout]; change accordingly. Check dilution factors. To change dilution factors, double click on samples (e.g., SPL 1) [Well ID] menu; [Type: Dilutions]. Enter dilution factor in SPL1:1. [OK]. Check that the microplate is preheating to 37°C.

7. Pipet, in duplicate, 5 μL of each SAMPLE into respective assigned wells (**Figure 1**). Pipet 5 μL of each CONTROL, BLANK (18MΩ H_2O), and NEFA Working STANDARD into appropriate microwells. *Change pipet tips between every sample, do not rinse the pipet tip in the sample, and pipet directly to the bottom of the well for best results.*

8. Using a multichannel pipet with 300-μL tips attached, pipet 170 μL of **Working Reagent A** into all test wells. Check for bubbles in the wells. Pop any bubbles with a small hypodermic needle (e.g., 26 g x ½ inch). *To prevent bubbles, use only room temperature reagent, and pipet at a slight angle.*

9. To load the plate into the microplate reader, press the green triangular [Read] button on the top menu bar of the open Synergy Gen5 2.04 protocol file. The door to the microplate chamber should open. Place the microplate into the plate reader with well A1 on the upper left-hand side. Follow computer screen prompts. The instrument will perform the protocol: shake for 15 s, delay at 37°C for 5 minutes, Read:1 at 550 nm. The microplate reader will then eject the plate, and a message "*Add Reagent B*" will appear on screen.

10. Using a multichannel pipet with 300-μL tips attached, pipet 85 μL of **Working Reagent B** into each well and return plate to microplate reader. Click [OK]. Reader will then continue the protocol: shake for 15 s, delay at 37°C for 5 minutes, Read:2 at 550 nm.

11. Results appear on the screen. The screen will prompt [SAVE]. Follow the guidelines below for editing/deleting points. Only edit results on [Plate 1] [Matrix] tab, [Data: Corrected Values] dropdown menu.

a) On [Plate 1] [Matrix] tab, [Data: Corrected Values] dropdown menu, check that the BLANK values are at or near zero and are consistent. [Mask] any BLANK that does not meet these criteria.

b) Check the standard curve (*Raw OD 550 Read 1 and Read 2 have been transformed as follows: Blank Transformation of both Read 1 and Read 2 to correct for blank reading, the linear regression is calculated from the NEFA Working Standard concentrations vs Blank OD550 Read 1 subtracted from Blank OD550 Read 2*). [Plate 1] [Graphs] tab, [Results: StdCurve Fitting Results]. Is the R^2 between 0.995 and 1.00? Are the variables (slope and intercept) similar to previous NEFA assays? If not, delete the bad points to get the best and most consistent standard curve. To do this, go to [Plate 1] [Matrix] tab,

[Data: Corrected Values] dropdown menu. Click on [Mask] button and then click on values to be masked. [Apply changes] [Close] [Yes].

c) To check standard differences (*assigned values vs values calculated and assigned based on the linear regression curve*) go to [Plate 1] [Statistic] tab, [Data: Concentration] dropdown menu. Compare standard values in Conc/Dil column with those in the Mean column. Are these values acceptable? If not, go back and [Mask] the appropriate standard values.

d) Is the CONTROL within control limits? Go to [Plate 1] [Statistics] tab, [Data: dilution factor calc] dropdown menu.

e) Do the SAMPLE values fall within the standard curve values? Go to [Plate 1] [Statistics] tab, [Data: dilution factor calc] dropdown menu. If not, repeat with different dilutions.

f) Are the SAMPLE values in normal range and/or what you expected?

g) To check CV, go to [Plate 1] [Statistics] tab, [Data: dilution factor calc] dropdown menu. Are the CV (%) of the SAMPLE values ≤ 7%? If not, repeat the analysis on that sample.

12. [Save] the .exp file in the [Project] folder.

13. Export the results to Excel (Paper with blue arrow Icon on top menu bar). Add identifying info to the report (date, tech initials, experiment name, type of sample, and catalog and lot numbers of all reagents used), [SAVE] the .xlsx file in the [Project] folder, and PRINT. Fill out an *NEFA HR Assay QC* sheet using the example below (**Figure 2**). Save QC files to the specific [Project] folder.

14. When finished using the microplate reader, close out of the program, and turn off the microplate master switch. Shut computer down at the end of the day, or when completed if you are the only user.

Repeat analysis on sample duplicates with CV greater than 7%.

Repeat analysis on samples that are greater than 1000 µmol/L after the appropriate dilution in saline.

Linearity: at least 1000 µmol/L.

Conversion 1000 µmol/L = 1000 µEquiv/L

Assay: Fujifilm Wako NEFA						Date/Tech: 9-11-20 MR		
Project: Carrington Soybean Hull Serum								
In-house pooled control made from bovine serum 9-8-20								
Average	506.53							
1SD	32.46		In-house range:					
2SD	64.91		441.61	to	571.44	µmole/L		
3SD	97.37							
							Linear regression	
Date	Running Plate #	Control NEFA (µmole/L)	Average	%CV			Slope A	Intercept B
9/9/2020	1	470.151	456.665	4.176			0.000226	-0.000980
	1	443.179						
9/9/2020	2	510.393	514.391	1.099			0.000225	-0.006650
	2	518.389						
9/10/2020	3	505.741	493.106	3.624			0.000218	-0.003220
	3	480.470						
9/10/2020	4	522.804	525.446	0.711			0.000227	-0.008570
	4	528.088						
9/10/2020	5	551.586	549.923	0.428			0.000210	-0.004470
	5	548.259						
9/10/2020	6	523.384	515.0765	2.281			0.000223	-0.006550
	6	506.769						
9/11/2020	7	491.139	482.073	2.660			0.000226	-0.004160
	7	473.007						
9/11/2020	8	521.218	522.5495	0.360			0.000225	-0.007960
	8	523.881						
9/11/2020	9	508.155	470.6795	11.260			0.000216	-0.003230
	9	433.204						
9/11/2020	10	527.069	535.373	2.194			0.000217	-0.009350
	10	543.677						
average			506.528			*average*	0.000221	-0.005514
stdev			32.457			*stdev*	0.000006	0.002715
%CV			6.41	2.88		*%CV*	2.57	-49.25
			%CV inter (between) assay	**%CV intra (within) assay**				

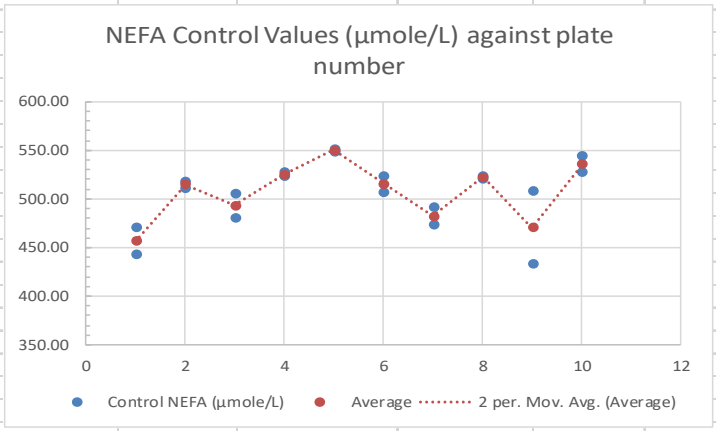


Figure 2. Example of QC Control Sheet for the Fujifilm NEFA assay.

FUJIFILM

Code No. 999-34691
991-34891
993-35191

HR Series NEFA-HR (2)

(ACS·ACOD method)

Intended use

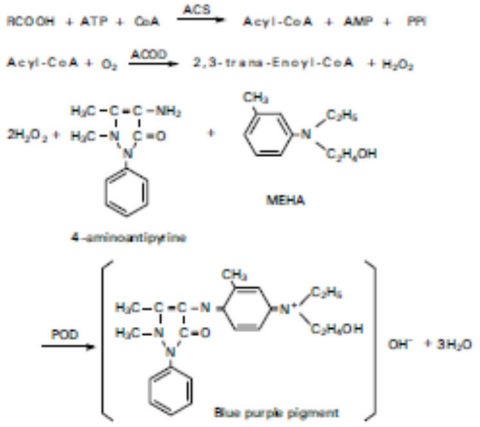
The HR Series NEFA-HR (2) is an in vitro enzymatic colorimetric method assay for the quantitative determination of non-esterified fatty acids (NEFA) in serum.

Summary and explanation of the test

Extraction methods are widely used for the colorimetric determination of non-esterified fatty acids (NEFA) in serum. NEFA are converted to their copper salts that are extracted into an organic solvent. The salts are then complexed with a dye for the purpose of colorimetric measurement [1,2,3,4]. Alternatively, extracted NEFA are titrated with standard alkali to an acid-base indicator endpoint [5,6]. These approaches are time consuming, hazardous and not easily automated. Wako has made extensive studies of NEFA quantitation and has succeeded in developing an original enzymatic method which is accurate, precise, simple and fast. The need for an extraction step has been eliminated and the method can be automated.
The Wako enzymatic method relies upon the acylation of coenzyme A (CoA) by the fatty acids in the presence of added acyl-CoA synthetase (ACS). The acyl-CoA thus produced is oxidized by added acyl-CoA oxidase (ACOD) with generation of hydrogen peroxide. Hydrogen peroxide, in the presence of peroxidase (POD), permits the oxidative condensation of 3-methyl-N-ethyl-N-(β-hydroxyethyl)-aniline (MEHA) with 4-aminoantipyrine to form a purple colored product which can be measured colorimetrically at 550 nm.

Principle of the method

Non-esterified fatty acids (NEFA) in serum, when treated with acyl-CoA synthetase (ACS) in the presence of adenosine triphosphate (ATP) and CoA, form the thiol esters of CoA known as acyl-CoA along with the byproducts adenosine monophosphate (AMP) and pyro-phosphate (PPi). In the second portion of the procedure, the acyl-CoA is oxidized by added acyl-CoA oxidase (ACOD) to produce hydrogen peroxide which in the presence of added peroxidase (POD) allows for the oxidative condensation of 3-methyl-N-ethyl-N-(β-hydroxyethyl)-aniline (MEHA) with 4-aminoantipyrine to form a purple colored end product with an absorption maximum at 550 nm. Hence the amount of NEFA in the sample can be determined from the optical density measured at 550 nm.



Reagents

(1) Color Reagent A
When reconstituted
0.53 U/mL Acyl-coenzyme A synthetase (ACS) (*Pseudomonas* sp.)
0.31 mmol/L Coenzyme A (CoA, Candida)
4.3 mmol/L Adenosine triphosphate (ATP) (*Bacterium* sp.)
1.5 mmol/L 4-aminoantipyrine
2.6 U/mL Ascorbate oxidase (pumpkin)
0.062% Sodium azide (as Color Reagent A Solution)
Store at 2-10°C.

(2) Solvent A
50 mmol/L phosphate buffer, pH 7.0
0.05% Sodium azide
Store at 2-10°C.

(3) Color Reagent B
When reconstituted
12 U/mL Acyl-coenzyme A oxidase (ACOD) (*Arthrobacter* sp.)
14 U/mL Peroxidase (POD) (horseradish)
Store at 2-10°C.

(4) Solvent B
2.4 mmol/L 3-methyl-N-ethyl-N-(β-hydroxyethyl)-aniline (MEHA)
Store at 2-10°C.

Warnings and precautions

(1) For in vitro diagnostic use.
(2) Not to be used internally in humans or animals.
(3) Do not use reagents past the expiration date stated on each reagent container label.
(4) Do not use the reagents described above for any purpose other than described herein.
(5) Do not use reagents which were frozen in error. Such reagents may give false results.
(6) If the reagents come in contact with the mouth, eyes or skin, wash off immediately with a large amount of water. Consult a physician if necessary.
(7) Color Reagent A and Solvent A contain sodium azide as a preservative (0.062% as reconstituted solution). Sodium azide may react with copper or lead plumbing to form explosive compounds. Even though the reagents contain minute quantities of sodium azide, drains should be well flushed with a large amount of water when discarding the reagents.
(8) The vials are sealed under vaccum. Slowly remove the stopper in order not to release the powder in the vial.
(9) Be careful not to cut yourself with the aluminium cap when removing it from the vial.

Physical or chemical indications of instability

The presence of precipitates in the reagents or values of control sera outside the manufacturer's acceptable range may be an indication of reagent instability.

Instruments

The reagent is designed to be used on commercially available automated analyzers such as Beckman SYNCHRON CX5® analyzer.
Refer to the operating manual for a description of instrument operation, specifications and calibration.

Specimen collection and preparation

Use serum as a specimen. Blood should be collected in the early morning after the patient has fasted for at least 12 hours. Collection of only a few mL of blood from the antecubital vein into a plain evacuated tube will be quite satisfactory.
After the blood has been allowed to clot, the serum should be separated by centrifugation as soon as possible. Please note that serum is the specimen of choice, but alternate collections may be acceptable.
Any specimen containing heparin is unsuitable for this analysis. Hence, any patient receiving heparin therapy, or any specimen collected in a heparinized collection vessel is unsuitable for this analysis.
Specimens that are noticeably icteric, hemolyzed or lipemic may yield inaccurate results unless a specimen blank is also analyzed.

Warning/Biohazard

It is recommended that specimen collection be carried out in accordance with NCCLS Document M29-T2. No known test method can offer complete assurance that human blood samples will not transmit infection. Therefore, all blood derivatives should be considered potentially infectious.

Procedure for Beckman SYNCHRON CX5®

Materials supplied

Refer to the section entitled "Reagents."

Materials required but not supplied

Beckman SYNCHRON CX5® analyzer
NEFA Standard Solution
Quality control material
All analyzer applications should be validated in accordance with NCEP and CLIA recommendations. For further assistance contact Wako Diagnostics Technical Service Department at 1-877-714-1924 or e-mail diagnostics@wakousa.com.

Reagent preparation

Color Reagent A Solution :
Add one bottle of Solvent A to one vial of Color Reagent A. Mix gently by inverting the vial until the contents are completely dissolved. Reconstituted solution is stable for one month at 2-10°C.

Color Reagent B Solution :
Add one bottle of Solvent B to one vial of Color Reagent B. Mix gently by inverting the vial until the contents are completely dissolved. Reconstituted solution is stable for one month at 2-10°C.

Test procedure

Parameter setting (Beckman SYNCHRON CX5®)

Reagent	HR Series NEFA-HR (2)
Test name	NEFA
Reaction Type	ENDPOINT2
Reaction Direction	Positive
Unit	mEq/L
Decimal Position	XXX
Calculation Factor	0
Math Model	LINEAR
Cal. Time Limit	9999hr
No. of Calibrator	2
Cal#1	saline/0.00/*2
Cal#2	Calibrator/*1/*2
Cal#3	
Cal#4	
Cal#5	
Cal#6	
Primary Wavelength	560 nm
Secondary Wavelength	670 nm
Sample Volume	4 μL
Primary Inject R1	225 μL
Primary Inject R2	
Secondary Inject Reagent	75 μL
Add Time	592 sec.
Calibrators	
Multi point Span 1-2	0.00
2-3	
3-4	
4-5	
5-6	
6-1	
RGT Blank Start Read	528 sec.
End Read	560 sec.
Low/High Abs	−1.5/1.5
Reaction Start Read	576 sec.
End Read	608 sec.
Low/High Abs	−1.5/1.5
Usable Range Low/High Limit	0/9999
Substrate Depletion	
Initial Rate	99.999
Delta Abs.	1.5

*1 : Input the assigned value of the calibrator.
*2 : Input the position of the calibrator.

Results

The final results are automatically calculated and printed in concentration. The results are given in mEq/L.

Calibration

The HR Series NEFA-HR (2) assay must be calibrated using the NEFA Standard Solution.

Quality control

A quality control program is recommended for all clinical laboratories. The analysis of control material in both the normal and abnormal ranges with each assay is recommended for monitoring the performance of the procedure. The values obtained for controls should fall within the manufacturer's acceptable ranges. If values are to be established for unassayed control material, the laboratory should assay each level of control material a sufficient number of times to generate a valid mean and acceptable range.

Limitations of the procedure

The linearity of HR Series NEFA-HR (2) is 0.01-4.00 mEq/L. If the NEFA value exceeds 4.00 mEq/L, dilute the sample 1 + 2 with saline, repeat the assay, and multiply the result by 3.

Expected values

The expected normal range for serum NEFA from fasting patients is 0.1 - 0.6 mEq/L. Since expected values are affected by age, sex, diet and geographical factors, each laboratory should establish its own expected values for this procedure.

Performance characteristics

Accuracy

The accuracy of this method was demonstrated by a recovery study.

No.	Added value	Expected value	Measured value	Obtained value	Recovery
	(mEq/L)	(mEq/L)	(mEq/L)	(mEq/L)	(%)
1	0.15	0.42	0.42	0.15	100.0
2	0.30	0.57	0.58	0.31	103.3
3	0.59	0.86	0.89	0.62	105.1

No.	Added value	Expected value	Measured value	Obtained value	Recovery
	(mEq/L)	(mEq/L)	(mEq/L)	(mEq/L)	(%)
1	0.39	1.43	1.43	0.39	100.0
2	0.65	1.69	1.69	0.65	100.0
3	0.78	1.82	1.82	0.78	100.0

No.	Added value	Expected value	Measured value	Obtained value	Recovery
	(mEq/L)	(mEq/L)	(mEq/L)	(mEq/L)	(%)
1	0.59	2.61	2.62	0.60	101.7
2	1.18	3.20	3.16	1.14	96.6
3	1.77	3.79	3.87	1.85	104.5

Precision

Within-run precision

Sample #	Replicates	Mean (mEq/L)	SD	CV (%)
1	20	0.51	0.0038	0.75
2	20	0.96	0.0059	0.61

Total precision

Number of assay days	Mean (mEq/L)	S_{WT}	S_T	CV (%)
20	0.548	0.0015	0.0041	0.75
20	1.082	0.0053	0.0531	4.91

Sensitivity

The minimum detectable level of this method is estimated to be 0.0014 mEq/L.

Correlation

A group of 97 serum samples with NEFA concentration ranging from 0.10 to 1.73 mEq/L was assayed by the described procedure and by a commercially available method. Comparison by values yielded a correlation coefficient of 0.992 and the regression equation was y = 1.027x + 0.041.

Specificity (Beckman SYNCHRON CX5®)
(Additive Study)

Hemoglobin (mg/dL)	None	100	200	300	400	500
NEFA (mEq/L)	0.48	0.47	0.45	0.44	0.42	0.40

Ascorbic acid (mg/dL)	None	10	20	30	40	50
NEFA (mEq/L)	2.16	2.15	2.14	2.13	2.14	2.11

Free Bilirubin (mg/dL)	None	10	20	30	40	50
NEFA (mEq/L)	1.74	1.69	1.65	1.60	1.57	1.56

Conjugated Bilirubin (mg/dL)	None	8	16	24	32	40
NEFA (mEq/L)	2.08	1.98	1.87	1.77	1.67	1.56

References

(1) Duncombe, W. G. : Clin. Chim. Acta 9. 122 (1964).
(2) Itaya, K., and Ui, M. : J. Lipid Res. 6, 16 (1965).
(3) Novak, M. : J. lipid Res. 6 431 (1965).
(4) Elphick, M. D. : J. Clin. Pathol. 21, 567.
(5) Trout, D. L., Estes, Estes, E. H. and Friedbekg, S. J. : J. Lipid Res. 1 : 199 (1960).
(6) Dole, V.P. and Meinertz, H. : J. Biol. Chem. 235, 2595 (1960).

Ordering information

Code No.	Product	Package
999-34691	HR Series NEFA-HR (2) Color Reagent A	4 × for 50 mL
995-34791	HR Series NEFA-HR (2) Solvent A	4 × 50 mL
991-34891	HR Series NEFA-HR (2) Color Reagent B	4 × for 25 mL
993-35191	HR Series NEFA-HR (2) Solvent B	4 × 25 mL
997-76491	Wako NEFA Linearity Set	10 mL
276-76491	NEFA Standard Solution	4 × 10 mL

Revision date ; Apr. 1, 2018

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18.03.19K04

Addendum Figure 1 (cont). Automated NEFA method 2018.