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INSTRUCTIONS FOR USE

Description	Part numbers	Hazard Symbols
Ultra Physiological Fluid Kit Ultra Physiological Routine Kit	80-2117-77 80-6000-06	
Biochrom Lithium Buffer Start-up Kit Biochrom Lithium Buffer Routine Kit	80-6000-16 80-6000-17	
Ultra Ninhydrin Kit 2L Ultra Ninhydrin Kit 8L	80-2118-30 80-2117-76	
As sold individual items: Lithium Buffer 1 Lithium Buffer 2 Lithium Buffer 3 Lithium Buffer 4 Lithium Buffer 5 Lithium Regeneration Buffer 6	 80-2038-15 80-2038-16 80-2099-83 80-2097-18 80-2037-69 80-2038-20	 None None None None None 
EZ Nin Reagent Kit 2L EZ Nin Reagent Kit 4L EZ Nin Reagent Kit 8L	80-6000-13 80-6000-14 80-6000-15	

Dansk	Oversatte versioner af dette dokument findes på	www.biochrom.co.uk/ userdownloads
Nederlands	Vertaalde versies van dit document zijn te vinden op	
Français	Vous trouverez les versions traduites de ce document sur	
Deutsche	Übersetzte Versionen dieses Dokuments finden Sie auf	
Italiano	Le traduzioni di questo documento sono disponibili sul sito web	
Lietuviškai	Šio dokumento vertimus galite rasti	
Español	Puede encontrar versiones traducidas de este documento en	
Svenska	Översätta versioner av detta dokument finns på	
Ελληνικά	Οι μεταφρασμένες εκδόσεις αυτού του εγγράφου μπορούν να βρεθούν στο	

(With particular reference to PKU)

DISCLAIMER
These chemical kits are intended solely for use in Biochrom Bio 30+ amino acid analysers. Not all components may be needed to perform testing: the term Device is used to refer to any functional ensemble of sub-components from the list above.
Use of these kits in other instrumentation is not covered within these instructions for use.

CE 2797

Revision History

Revision No	Issue date	Notes
1	24/02/2004	First Release
2	21/04/2004	Re-written to conform with IVD requirements
3	04/05/2006	Reviewed for technical changes
4	23/02/2007	Declaration of Conformity amended to cover complete kit and requirements of LRQA. Issue number removed from D of C
5	01/02/2008	MD signature updated
6	01/06/2011	MD signature updated, new reference to 80-6000-06 chemical kit added, reference to Bio30+ added.
7	10/09/2012	Buffer names updated
8	04/03/2013	Formatting changes
9	19/05/2014	New chemical kits added: 80-6000-16 and 80-6000-17. EZ Nin Reagent part numbers added. Introduction of OpenLAB software. Certificate of Conformity updated.
10	5/11/2015	Change in address from 22 Cambridge Science Park to 1020 Cambourne Business Park CB23 6DW UK
11	16/11/2015	Updated text in Section 1. Intended Use 5.2.2.1 EZNin shelf life extended to 2 years
12	15/03/2016	Change to Sec. 11.2 to state clearly certain sample types are not recommended. Additions to Sec. 14.1 and 14.2 addressing the upper operational limits.
13	07/07/2016	Table in Sec. 2 modified and references *27* and *28* added to support the statement. Add section 11.4 to describe the deproteinisation of urine samples. Modify section 12.1 to include urine samples Change the Ultraninhydrin shelf-life in section 5.2.1.2 Additional comments on handling EZ Nin Reagent section 5.2.2.2
14	27/07/2016	Minor typographical correction in section 11.4
15	13/10/2016	Clarification of the Intended use of Device Added amended Declaration of Conformity.
16	30/01/2018	Formatting changes Adding translation on front cover directing to language versions of this document on the Biochrom website EZNin shelf life extended to 3 years
17	02/09/2019	Revision against the requirements in other jurisdictions. Addition of individual buffers and reagents to the description with Hazard warning symbols where applicable.
18	30/10/2020	Update company address. Update hazard symbol information to match update of MSDS documents. Added the updated DofC and the new notified body number.
19	27 APR 2022	Removal of Declaration of Conformity from Instruction for Use. Declaration of Conformity provided as a separate document. Removal of obsoleted device references.
20	05 JAN 2023	1. Section 1 Intended Use - Added 's' to amino acid and 'and tyrosine' for completeness purposes. 2. Section 3.3.1 Chemistry - typographical correction from 'imino' to 'amino'.

		3. Section 4.4 Purpose of the Device - Added ', namely phenylalanine and tyrosine'. 4. Section 10.1 is updated to remove relevant details on Urine sample.. 5. Section 10.2 Warning concerning other sample types - Removed 'A decision…' and sections 10.2.1 and 10.2.2. 6. Section 10.3.1 Precautions is removed. I have removed this as this implies that we have performed some anticoagulant studies. 7. Section 10.3.1 Pre-analysis Treatment - Removed 'Calibration standard' and replaced with 'Reference standard' 8. Section 10.4 Deproteinisation of Urine Samples is removed 9. Section 11.1 Injection conditions - Removed details on urine sample. 10. Section 12.2 Calibration standards - changed wordings to remove association of a 'Calibrator'. 11. Section 14.6.3 removed extra bullet point
21	31 MAR 2023	1. Instructions for use product content table – product names aligned to device name as referenced on Notified Body EC certificate. 2. Typographical corrections applied throughout document.

CONTENTS

1 Intended use of the Device

Biochrom Lithium Buffer Kits and Ninhydrin Reagent Kits (Ultra Ninhydrin Kits and EZ Nin Reagent Kits), are intended solely for use on the Bio 30+ series of Amino-Acid Analysers. They are for *in-vitro* diagnostic use and are intended to perform quantitative analysis of the amino acids phenylalanine and tyrosine in de-proteinised blood plasma as an aid in diagnosis and monitoring of the metabolic disease Phenylketonuria.
Test results are not intended to be sole diagnostic tool and must be interpreted in context with other laboratory and clinical findings.

There are no known contraindications for the use of these kits.
Users are reminded that some drugs and drug metabolites may generate additional peaks.

2 Description of Phenylketonuria

Direct or indirect disorders of phenylalanine metabolism cause hyperphenylalaninemia, generally known as phenylketonuria (PKU), with elevation of phenylalanine above the normal ranges shown below. PKU is one of the most studied inborn errors of metabolism, affecting 0.006% of births. Phenylalanine, an essential amino acid, is normally hydroxylated to tyrosine by the enzyme phenylalanine hydroxylase (PAH).
Classical hyperphenylalaninemia (PKU I) is caused by a deficiency of PAH. PAH activity is less than 2% of normal. Besides phenylalanine high urinary excretion of phenylpyruvic acid, phenyllactic acid, phenylacetic acid, mandelic acid and phenylglyoxylic acid can be detected by organic acid analysis. PKU is clinically expressed in mental retardation, behavioural abnormalities, dermatological abnormalities, hypopigmentation, and impaired physical growth affecting head circumference and height. The clinical symptoms of PKU are now of mainly historic interest, because the disease is prevented by neonatal screening.
Other types of PKU are the result of an impaired synthesis of the cofactor BH4. In earlier times these types of PKU were called malignant PKU, because despite adequate treatment a progressive neurological disease developed. In PKU II dihydropteridine reductase (DHPR) is deficient. Patients lacking DHPR are deficient in the neurotransmitters whose synthesis depends on the normal activity of DHPR-dependent tyrosine and tryptophan hydroxylases.
Treatment of these patients exists in dietary control of the hyperphenylalaninemia, and in restoration of neurotransmitter homeostasis by oral administration of L-dopa, 5-OH-tryptophan, and other agents.
Another phenomenon in the hyperphenylalaninemia syndromes has become maternal PKU. If the intrapartum phenylalanine level exceeds 1.1mmol/l foetal pathology can occur, leading to intrauterine growth delay, congenital heart malformation, mental retardation and microcephaly. The reason for foetal pathology is that in the foetus the blood phenylalanine level is 1.13-2.91 times higher than in maternal blood. As a consequence it is necessary that the female PKU patient has to use a PKU corrective diet again before she is pregnant. For female PKU patients who are off the diet this is sometimes very difficult. Hyperphenylalaninemia can secondarily occur in the new-born, if the mother is an untreated PKU patient, in primary or secondary tyrosinemia, and in a high neonatal protein intake.

Amino acid concentrations in normal plasma (micromoles/L) for various age ranges

Amino acid	Men	Women	Adolescents	Children
	(n = 50) [27]	(n = 15) [27]	(n = 80) [28]	(n = 52) [28]
Phenylalanine	46–74	42–62	34–86	26–98

3 Principle of analysis for phenylalanine and other amino acids

3.1 Outline Operation of the Biochrom Amino Acid Analyser

The Biochrom AAA amino acid analyser is a PC controlled automatic liquid chromatograph with post column detection system, which is operated in conjunction with the Biochrom Lithium Buffer Kits and ninhydrin reagent kits (Ultra ninhydrin kits or EZ Nin Reagent kits).
The sample containing a mixture of amino acids is injected from a temperature controlled autosampler onto a column of cation exchange resin. Buffers of varying pH and ionic strength are then pumped through the column to separate the various amino acids. The column temperature is accurately controlled and adjusted automatically as necessary to produce the required separation. The column eluent is mixed with the Ultra Ninhydrin Reagent or the EZ Nin Reagent™, this mixture being passed through the high temperature reaction coil, maintained at 135°C (138ºC when using EZ Nin Reagent). In the reaction coil, the ninhydrin reacts with the amino acids present in the eluent to form conjugated compounds with absorbance maxima at wavelengths of 570 and 440nm. The amount of each reaction product is directly proportional to the quantity of amino acid present.
From the reaction coil the eluent /ninhydrin mixture is pumped to the photometer unit, which measures the amount of light absorbed at 570nm and 440nm.
The photometer output is connected to a two-channel computer-based integration system, one channel for the 570nm output, the other channel for the 440nm output. The amino acid concentrations are recorded as a series of peaks. The retention time of the peak identifies the amino acid, the area under the peak indicating the quantity of amino acid present. As the amino acid analyser is a comparative instrument, a calibration analysis must be performed before commencing a series of analyses thus producing a standard trace for comparison purposes.
After each sample analysis, the column is regenerated by pumping a strong base through the column followed by equilibrating buffer, which adjusts the analytical column resin to its correct pH prior to the next analysis.

3.2 Theory of Ion Exchange Chromatography

Amino acid analysis is achieved by a cation exchange chromatography process. The resin has a negative charge and the amino acids are added at a low pH, which ensures they are all positively charged. The conditions on the column are then altered to increase the pH, the temperature and the concentration of the buffer counter ions. These changes result in the iso-ionic point of an amino acid being reached, at which point the ionic attraction to the resin is lost and the amino acid elutes from the column.
The ion exchange process can be represented as:

MATRIX----------SO_3 – Na < > MATRIX ----------SO_3 – NH_2 – R

Separation is effected by increasing the pH or the counter-ion concentration, so moving the equilibrium towards the left.
The resin used in amino acid analysers consists of spherical beads of polystyrene. The polystyrene chains are cross-linked by the incorporation of divinylbenzene groups (DVB) and are also sulphonated to give them a negative charge. The structure of the resin consists of chains of styrene units cross-linked with divinylbenzene.
The cross-linked resin structure is the matrix to which are bonded the charged groups ($-SO^-_3$). Ion exchange takes place by the counter-ions (the interchangeable ions which are attached to the matrix by heteropolar bonds i.e. the positively charged groups in the buffers or on the amino acids contained in the buffer) diffusing into the matrix and changing places with ions which are loosely attached there.

Production of resins is based on emulsion polymerisation in these stages:

- Reaction of styrene and divinylbenzene while mixing under pressure and elevated temperatures with initiators.
- Separation and purification of polymer beads.
- Sulphonation of polymer beads at elevated temperature for several hours.
- Isolation of beads within required size range followed by extraction and cleaning.

The variable parameters in resin used for amino acid analysis are particle size, degree of sulphonation and degree of cross-linking. Optimal loading of active sites is 5meq/g which gives a capacity of 1.155×10^{14} molecules/g of resin.

3.3 Detection and analysis with ninhydrin reagent

3.3.1 Chemistry

Ninhydrin is a powerful oxidising agent and elicits the oxidative deamination of the a-amino group, liberating ammonia, carbon dioxide, an aldehyde with one less carbon atom and a reduced form of ninhydrin, hydrindantin. The ammonia then reacts with the hydrindantin and another molecule of ninhydrin to yield a purple substance (Ruhemann's purple) that absorbs maximally around 570nm. This absorbance is a linear function of the amount of a-amino groups present, and this reaction provides a convenient and quantitative colorimetric assay for all organic compounds with a-amino groups. The reaction is accelerated at high temperatures and Biochrom analysers incorporate a thermostated post-column reactor system which has been optimised to control reaction conditions at 135^{O}C or 138ºC depending on the reagent used, and for very short times. This enables stable and reproducible detection.

Hydrindantin is needed in the detection reagent to prevent a side reaction that would reduce the amount of Ruhemann's purple formed.

In the reaction with the imino acids, such as proline and hydroxyproline, which do not have free a- amino groups, the product is a bright yellow compound which is monitored at 440nm. Measurement at any wavelength below 440nm would give a higher response but a significant part of that response would be due to the absorbance of unreacted ninhydrin, therefore any measurements at these wavelengths would not be a true measurement of the amount of amino acid originally present.

The Beer-Lambert Law defines the relationship between absorbance, transmittance and molar concentration as:-

$$A = \log_{10} \frac{Io}{I} = \log_{10} \left(\frac{100}{T}\right) = Ecb$$

where

A = absorbance

T = transmittance

Io = intensity of the incident light

I = intensity of the transmitted light

E = molar absorptivity ($dm^3\ mol^{-1}\ cm^{-1}$)

c = molar concentration ($mol\ dm^{-3}$)

b = path length (cm)

By the Beer-Lambert Law a linear relationship between concentration and absorbance is expected. For photometric detection this relationship is only valid up to approximately 2 absorbance units. Above approximately 2A the deviation from linearity means that calibration graphs should be used for quantitation.

4 Instrumentation parameters

4.1 Name and address of the manufacturer

Biochrom Ltd, Unit 7, Enterprise Zone, 3970 Cambridge Research Park, Waterbeach, Cambridge. CB25 9PE, UK

4.2 Identity of Device and contents of the packaging

Biochrom Amino Acid Analysers Ref# 80-6000-50, -51, -52, -53, -54, -55, -56, -57 and -58 with Biochrom Lithium Buffer Kits 80-6000-16 and -17 and Ultra Ninhydrin Kits 80-2118-30 and 80- 2117-76 or EZ Nin Reagent Kits 80-6000-13, 80-6000-14 and 80-6000-15.

4.3 Ambient conditions

Samples and reagents should be kept free from atmospheric and particulate contamination. Sterile conditions are not required. The device should be operated between 10-25ºC without excess humidity. Adequate bench space and ventilation are required.

4.4 Purpose of the Device

The kits and the equipment are intended primarily for the quantitative analysis of amino acids, namely phenylalanine and tyrosine in de-proteinised blood plasma as an aid in diagnosis and monitoring of the metabolic disease Phenylketonuria. They are for *in vitro* use only.

4.5 Storage and handling conditions

Reagents should be stored and handled as described on the kit labels and as described in the following section on reagents.
The Analyser requires the following handling facilities:

4.5.1 Mains Supply
The Mains supply required by the instrument is in the 100-240VAC range. At least four mains electrical outlets should be available: one each for the main unit, for the computer for the printer and for the autosampler. If excessive disturbance to the mains voltage is anticipated, a mains stabiliser should be used.

4.5.2 Nitrogen Gas Supply
Oxygen free nitrogen gas, 99.99% purity is required, with regulation to 5 bar, 75 p.s.i. The nitrogen line supplied has a ¼" BSP fitting.

4.5.3 Drainage
The waste products produced by the instrument are classed as harmful. It is recommended that waste material is disposed according to local, state or federal regulations. The waste bottle should have at least 10 litre capacity.

4.5.4 Special Considerations
The Amino Acid Analyser must be used under analytical laboratory conditions with additional environmental considerations. Although normal clean laboratory conditions are usually quite satisfactory, it is useful to consider some special points to which attention should be paid: Atmospheric conditions should be relatively free of ammonia and carbon dioxide to avoid interference with measurement of amino acids. This is very important when working at high sensitivity.

4.6 Safety

Instructions and warnings should be observed as indicated on the product labels. More detailed information can found on the Safety Data Sheets which may be downloaded from the company website here: http://www.biochrom.co.uk/msdssearch.asp or supplied upon request. Information on the safe use of the instrument may be found in the User Manual supplied.

If it is necessary to move the Device between locations the chemical kits should be transported in their original packaging and accompanied by the original labels together with any enclosed paperwork.

Whilst every effort has been made to design out the risks associated with using the device there are Residual Risks. These arise as a result of an irreducible minimum in the type and concentration of the active components.

4.7 Use of the Device

The Device is not intended for self-testing. It should be used only by trained professional users.

5 Chemical Requirements

5.1 Lithium Buffers

5.1.1 Shelf life

The storage conditions, shelf life and batch numbers are shown on the label for each kit component. The buffers are stable for 3 years from manufacture and should be kept at room temperature (15-25ºC) out of direct sunlight.
Buffers that are partially consumed may be used up to the expiry date provided they are kept in the original container, is properly re-sealed and is stored as described above.

5.1.2 Composition and concentration of active ingredients

There are 6 chromatography eluents and 1 loading buffer making up the Lithium Buffer Kits:

80-2038-15	Lithium buffer 1	pH 2.80, 0.2M
80-2038-16	Lithium buffer 2	pH 3.00, 0.3M
80-2099-83	Lithium buffer 3	pH 3.15, 0.5M
80-2097-18	Lithium buffer 4	pH 3.50, 0.9M
80-2037-69	Lithium buffer 5	pH 3.55, 1.65M
80-2038-20	Lithium Regeneration 6	0.3M
80-2038-10	Lithium Loading Buffer	pH 2.20, 0.2M

These solutions are installed directly in the Analyser buffer reservoirs 1 to 6 respectively (except for the loading buffer)
The following table details the composition of the buffers:

Component	Buffer 1	Buffer 2	Buffer 3	Buffer 4	Buffer 5	Buffer 6	Loading Buffer
Lithium hydroxide	Yes	Yes	Yes			1.3%	
Phenol	0.1%	0.1%	0.1%	0.1%	0.1%		0.1%
Thiodiglycol	0.2%	0.2%	0.2%	0.2%	0.2%		0.2%
Propan-2-ol	1.5%	1.5%					
Hydrochloric acid	1.6%	1.5%	1.4%				1.8%
Other	Citric acid	Citric acid Lithium chloride	Citric acid Lithium chloride	Citric acid Lithium chloride	Citric acid Lithium chloride		Citric acid Lithium chloride

5.2 Ninhydrin detection reagents

5.2.1 Ultra Ninhydrin Reagent Kits

5.2.1.1 Shelf Life

The storage conditions, shelf life and batch numbers are shown on the label for each kit component. Ultra Ninhydrin Kit is stable for 3 years from manufacture when unopened and should be kept at room temperature (15-25ºC) out of direct sunlight.

The kit should be consumed in its entirety, as per the instructions below, and never split and stored in fractional parts.

5.2.1.2 Composition and instructions for preparation

Ultra Ninhydrin reagent is 1% ninhydrin, 0.08% hydrindantin in 5M aqueous acetate/ethanediol. The Ultra Ninhydrin reagent kits (80-2118-30 and 80-2117-76) are supplied as 2 components, ultra ninhydrin solution (80-2117-64) and Ultrosolve plus (80-2117-65), and makes 2L of final reagent. It is prepared before use as follows:

1. Remove reagent bottle from Analyser.
2. Add Ultrosolve Plus into Biochrom Amino Acid Analyser reagent bottle, retaining about 50 ml for rinsing ninhydrin reagent.
3. Bubble Ultrosolve Plus in the reagent bottle with oxygen-free nitrogen for 10 minutes, while mixing with a magnetic stirrer.
4. Shake Ultra Ninhydrin solution well and add to the Ultrosolve Plus solution, under nitrogen while continuing to stir.
5. Add the remaining Ultrosolve Plus to the Ultra Ninhydrin reagent bottle, rinse and add to the reagent mixture.
6. Continue to bubble nitrogen through the completed reagent and stir for another 10 minutes before installing on the instrument.

Ultra Ninhydrin reagent 2L bottle is sufficient for 120 hours of continuous operation of the Analyser and reagent remaining on the Analyser after 2 weeks should be discarded.

5.2.2 EZ Nin Reagent Kits

5.2.2.1 Shelf life

The storage conditions, shelf life and batch numbers are shown on the label for each kit component. EZ Nin Reagent Kit is stable for 3 years from manufacture when unopened and should be kept at room temperature (15-25ºC) out of direct sunlight.

Reagent that is partially consumed may be used up to the expiry date provided it is kept in the original container, is properly re-sealed and is stored as described above.

5.2.2.2 Composition and instructions for preparation

EZ Nin Reagent composition is as follows:

Concentration	Product Name	CAS Number	EC/EINECS
47.9%	Ethylene glycol [Acute Tox. 4]	107-21-1	203-473-3
29.5%	Water [Non Hazardous]	7732-18-5	231-791-2
8.0%	Potassium acetate [Non Hazardous]	127-08-2	204-822-2
6.4%	Acetic acid [Flam Liq. 3]	64-19-7	200-580-7
5.5%	Diethylene glycol methyl ether [Repr. 1A]	111-77-3	203-906-6
1.8%	Ninhydrin [Acute Tox. 4]	485-47-2	207-618-1
0.9%	Potassium formate [Non Hazardous]	590-29-4	209-677-9

The EZ Nin Reagent kits (80-6000-13, 80-6000-14 and 80-6000-15) are supplied as a single component being EZ Nin Reagent, part# 80-6000-12, and makes 2L of final reagent. No preparation is required prior to use on the Instrument.
The Analyser settings should be modified to obtain a default reaction coil temperature of 138ºC. EZ Nin Reagent 2L bottle is sufficient for 120 hours of continuous operation of the Analyser.

When Reagent is prepared from Ultraninhydrin and Ultrosolve Plus there are oxygen-sensitive materials present and consequently the product deteriorates from the moment of mixing. For EZ Nin Reagent this is not the case: the product on the instrument is simply the product in the plastic transport bottle. Therefore, the Reagent can be topped up directly onto the instrument without any precautions to exclude air. Users can expect the product placed on the instrument to function correctly within the stated Expiry Date on the bottle.

5.3 Materials required but not supplied

- Timed Microcentrifuge for 1.5ml conical tubes capable of 10000g for 5 mins
- 5-Sulphosalicylic acid, analytical grade
- Capped conical 1.5 ml polythene tubes
- Pipettes covering range 0-1000 microlitres and polythene pipette tips.

6 System obligations

6.1 Operating conditions

Before use the Biochrom AAA system should be installed by competent Biochrom trained personnel and have completed test analyses in accordance with the performance parameters required on manufacture.

6.2 User training

Users of the Biochrom AAA should have completed training in accordance with the Amino Acid Analyser Customer Training Course shown in the Biochrom training details and on the Biochrom website (www.biochrom.co.uk), either at Biochrom Ltd, Cambridge or on-site.

The course content is:

- Amino Acid Analysis & Biochrom AAA Theory.
- Sample handling and preparation/practical
- Types of Samples
  - Protein hydrolysates
  - Physiological fluids and related samples
- Chromatography optimisation
- Operation of Biochrom system – practical and overnight analyses
- Practical Interpretation and optimisation of data
- Data Handling -Practical reprocessing- Data export
- Routine maintenance
  - Fluidics – Pump -Photometer
  - Column maintenance
- Biochrom repacking service
- Buffers/ Ninhydrin Reagent QC, certification and web site details
- Application notes and web site
- Operation of screening analyses, accelerated chromatography
- Practical analysis and interpretation of typical samples

7 System Setup

7.1 Chromatography details

Operating conditions are set up on installation. Chromatography is carried out on a 200x4.6 mm column with buffer flow rate 25ml/hour and ninhydrin flow rate 20 ml/hr.
A typical program of chromatography conditions is shown below together with the chromatogram, although times and temperatures may differ slightly between Analysers to achieve peak separation within specification.

03:39:07 pm **BioSys** 23/04/2003

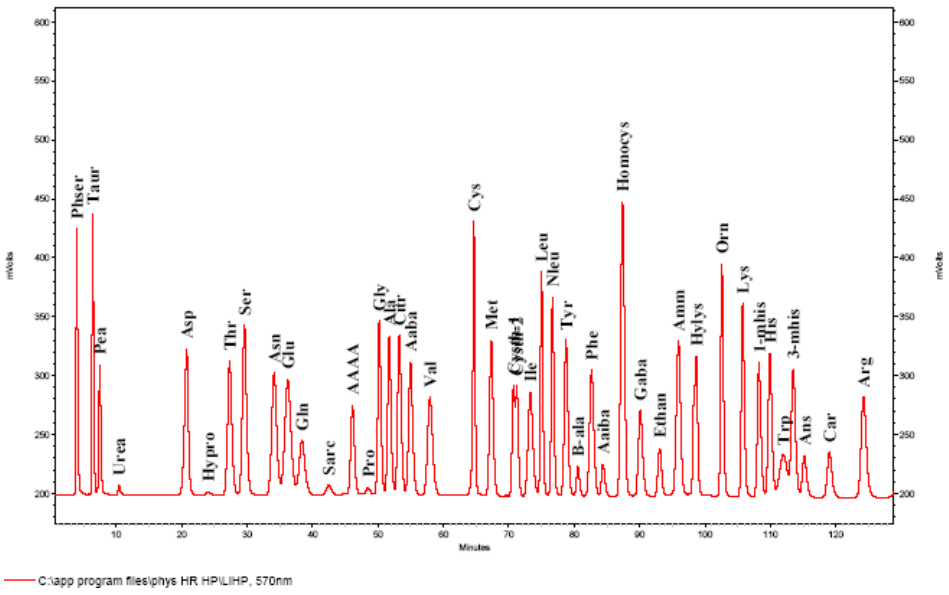
	Buffer	Molarity	pH	Batch No.
Buffer 1 -	Lithium citrate buffer	0.20	2.80	10385
Buffer 2 -	Lithium citrate buffer	0.30	3.00	10356
Buffer 3 -	Lithium citrate buffer	0.50	3.15	10370
Buffer 4 -	Lithium citrate buffer	0.90	3.50	10364
Buffer 5 -	Lithium citrate buffer	1.65	3.55	10372
Buffer 6 -	Lithium hydroxide solution	0.30		10383
Reagent	Ninhydrin			TR2
	Ultrosolve			TR2

Title: **Bio 30**
Filename: C:\app program files\phys HR HP\LiHR.prg
Comments: High resolution program

Nin Flow Rate: 20.0 ml/h

No.	Time	Temp	Buffer	Pump	Nin	Rec	Commands
1	01:00	33°C	1	20.0ml/h	ON	OFF	
2	00:00	33°C	1	20.0ml/h	ON	OFF	Reset
3	01:00	33°C	1	20.0ml/h	ON	OFF	Load
4	05:30	33°C	1	20.0ml/h	ON	ON	
5	38:30	33°C	2	20.0ml/h	ON	ON	
6	20:00	40°C	3	20.0ml/h	ON	ON	
7	09:00	64°C	3	20.0ml/h	ON	ON	
8	34:00	64°C	4	20.0ml/h	ON	ON	
9	50:00	80°C	5	20.0ml/h	ON	ON	
10	06:00	80°C	6	20.0ml/h	ON	ON	
11	06:00	80°C	1	20.0ml/h	ON	ON	

PHYSIOLOGICAL AMINO ACIDS HIGH PERFORMANCE SYSTEM



8 Operation of instrument software

a) Ensure the Bio AAA is switched on and all reagents and connections are installed.

b) Select the Biosys programmer icon from desktop shown in the list below.

c) Open file and load the default or selected sample list.

d) Select run. The Analyser then starts with the first program on its sample list. If the autosampler has been loaded as defined in the list, analysis continues automatically to the end or until intervention by the user. At the end of the sample list the Analyser shuts down automatically.

9 Programmer operation

Biochrom AAA chromatography is performed by programs. Each program is a sequence of steps which are specifically designed to control the flow of buffers and reagent through the system to complete an analysis.

Each step allows the column temperature, buffer, buffer pump flow rate and Ninhydrin pump to be controlled, as well as controlling the recorder and optionally performing a load or baseline reset. Each step is maintained for the period defined in the Time field. One program completes a single analysis and also regenerates and equilibrates the chromatography column ready for the next analysis.

The Ninhydrin pump rate can be set once for each program. Normally either one or no Sample Load is performed per program. This is now normally done at the start of each program, as opposed to previous versions, which implemented the load as the final step.
The program can also contain a title and a comments field, which can be used to identify the program.

Sequences of samples are run using programs linked together in a sample list.

The operation of Biochrom AAA chromatography is under control of the BioSys software. This consists of a number of individual applications, which link to provide a working system.
Each application is selected from a separate icon and 5 Main applications provide the functionality to set up and drive the BioSys system for the operator.

a) BioSys Set up
b) BioSys Editor
c) BioSys Programmer
d) BioSys Manual
e) BioSys Fluidics

Other System and Utility Applications provide a guide to current status and logs of samples and programs completed by the instrument.

The sample list is built up by addition of programs. These are called up from the appropriate directories using the Insert button.
Subsequently the buttons Insert, Delete and Edit on the right hand side are used to build up a list of programs to analyse each sample loaded.
The sample list is started by clicking on the Run button.

When the Analyser is in operation the current program steps and details are shown by opening the program window.
Completed steps are indicated by a tick mark on the left hand side of the window and the current step by a green arrow.

Current Status
Use the system settings from the controller to check the current status.
Check that reagent levels are adequate for the sample series via the control command and reagent usage
Check that buffer assignments are correct using the menu in the manual controller and enter the current descriptions as required.

Check and archive run and sample logs

During operation and after completion of an analysis the program and sample logs accumulate all operating details for archiving.

Check that these are filed to provide a complete record of samples analysed with their related control programs.

Before starting another series of runs check through the logs to ensure any relevant incidents have been actioned and all previous runs have been properly completed and recorded.

10 Data Handling Operation

10.1 OpenLAB/EZChrom Elite Setup

Configuration of the data handling system is carried out on installation
The following notes give the basic instructions for operation of data handling. Further detail is given in the on-line help pages of the data handling software or in the PDF User Manual.

10.1.1 Identification of peaks

- Open Software (double click on the icon) Launch offline instrument
- Go to File\method\open and select method
- Go to File\Sequence\open and select sequence Open data file,
- Click on the analyse symbol to analyse the data
- Right button mouse on the chromatogram for displaying annotations (green arrow to add, red arrow to remove)

10.1.2 Export of data

- Go to Method\advanced Tick Export enabled Select parameter to export Select path for export files
- Open sequence file and select files to process. Process sequence.

10.1.3 Custom reports

- Go to Method\custom report
- Right button mouse to insert field, sample id, user name, method name, print time
- Right button mouse to insert graph, data graph, data source, current data, trace (570nm) Do the same as above to 440 nm channel
- Right button mouse to insert report\
  run report File\methods\save
- Sequence process.

11 Sample collection and preparation

11.1 Notes on sample collection

Blood samples in the range 0.1 to 1.0 ml are taken from patients by the usual clinically proven safe methods of collection. Analyses are carried out on plasma subsequently centrifuged from blood samples, separately from the patient.

10.2 Warning concerning other sample types

Biochrom does not recommend the use of whole blood or blood serum as samples.

10.3 Deproteinisation of Plasma Samples

10.3.1 Pre-analysis Treatment

Plasma must be fully de-proteinised prior to analysis, the pH adjusted and then filtered to remove any remaining particulate material. There are several methods that could be used but the following method has been found to work well:

1. Collect whole blood directly into heparin coated tubes.
2. Centrifuge and separate, ensuring that the cellular buffy-coat is completely avoided.
3. Cool the separated plasma to4ºC.
4. Make up a 10% sulphosalicylic acid containing the required concentration of internal standard e.g. Norleucine or Amino Ethyl cysteine and mix this 1:1 with sample
5. Immediately mix the contents of the tube and allow to stand for 5min at 4ºC. Ensure that the precipitate has formed completely.
6. Spin down the precipitate, preferably at 4ºC using 10,000g at least, until the supernatant is clear. (If lipid is present in the sample, it will form a surface layer after centrifugation, therefore ensure that the pipette tip is under the liquid surface when the supernatant is removed, to avoid carry-over)
   The reference standard can be treated with sulphosalicylic acid in the same way as the sample to ensure that both are loaded for analysis at the same pH and give identical retention times for corresponding peaks. Otherwise if the loading pH is lower than the standard the retention times of the initial amino acids may be increased.
7. Filter the sample through a 0.2micron membrane filter prior to analysis on an Amino Acid Analyser.

12 Sample analysis

12.1 Injection conditions

Program the analyser to load 40 microlitres of de-proteinised plasma if the original sample has been diluted 1:1..

12.2 Replicate analyses

Inject duplicate aliquots from each de-proteinised sample and average these to report the final results.

13 Calculation of results

13.1 Calculation method.

The Biochrom AAA data handling software calculates peak areas for sample and calibration standard chromatograms. For phenylalanine peak areas are routinely calculated from the 570nm outputs.

Calibration data is derived from a known standard mixture as supplied with the Biochrom AAA system. Standard analyses should be analysed at the start and end of sample groups and bracketing calibration used to calibrate the data handling method.

13.2 Reference Materials

Reference standard and analytical integrity should be assessed. Pure amino acids are necessary for standardisation. Analyses have to be standardised with material of known value and monitored with stable QC samples preferably authenticated by collaborative analyses with other similar laboratories. Reference materials do not form part of the kit.

Standard mixtures should be checked as free from other contaminating chromatography peaks, which could interfere with calculation and correct assignment of peak areas. A pure single source of phenylalanine should be run separately to monitor new standard batches. Continuous records of standards and controls should be maintained to check for deviations of response.

13.3 Criteria for standardization

Standards should be in the linear response range as shown in the calibration report data output graph below.
It is recommended that standards are loaded at 5nmol on the column.

Use internal standard addition wherever possible. Recommended internal standards are norleucine, aminoethylcysteine and diaminobutyric acid. Use one of these in sample and standard to give a final concentration of 0.25mM in the injection vial.
If analytical sample measurements exceed the linear range and re-sampling is possible then the injection volume can be reduced. Reduction below 10µL may reduce the accuracy of the results and volumetric dilution of the sample may be preferable. If re-sampling is *not* feasible, the upper measurement range can be increased by calculation of peak areas from the analyser 440nm response.

The example of data output shows calibration of phenylalanine for PKU.

Control samples of known phenylalanine content should be measured with each group of samples. These should be maintained at or below –20 °C during storage to ensure sufficient stability.

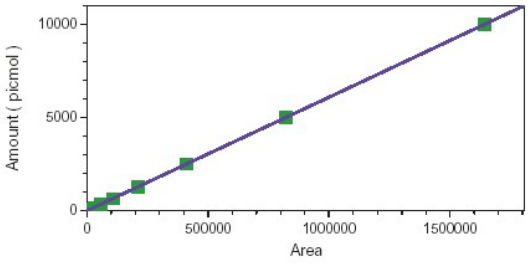
Participation in an external quality control scheme such as ERNDIM is recommended, see: https://www.erndim.org/home/qascheme.asp

Calibration Report

Method: G:\DATA\LabData\Common\linearity2\LINLI.MET
Print Time: 01/04/2004 10:42:34 am
User: System
Instrument: Biochrom 30 (Offline)

phe (570nm)
Average RF: 0.00533567 RF StDev: 0.00100319 RF %RSD: 18.8016
Scaling: None LSQ Weighting: None Force Through Zero: On
Replicate Mode: Replace
Fit Type: Linear
y = 0.00608348x + 0.000000
Goodness of fit (r^2): 0.999962

Peak: phe -- ESTD -- 570nm



	Level 2	Level 3	Level 4	Level 5	Level 6
Amount	9.766	19.5	39	78.125	156.25
Area	3712	4080	7656	14578	29135
RF	0.002630926	0.00477941176	0.00509404388	0.00535910275	0.0053629655
	66864395	470588	714734	757991	0540587
Last Area					
Residual	-12.8159	-5.32058	-7.57509	-10.5599	-20.9921
Rep StDev					
Rep %RSD					
Rep 1 Area	3712	4080	7656	14578	29135
Rep 1 User					
Rep 1 Data File					
Rep 1 Sample ID					
Rep 1 Calib. Time					

	Level 7	Level 8	Level 9	Level 10	Level 11
Amount	312.5	625	1250	2500	5000
Area	58270	106156	209566	411526	821134
RF	0.005362965	0.00588756170	0.00596470801	0.00607495030	0.0060891401
	50540587	164663	561322	690649	403425
Last Area					
Residual	-41.9841	-20.7974	-24.8896	-3.50838	4.65134
Rep StDev					
Rep %RSD					
Rep 1 Area	58270	106156	209566	411526	821134
Rep 1 User					

Calibration Report

Method: G:\DATA\LabData\Common\linearity2\LINLI.MET
Print Time: 01/04/2004 10:42:34 am
User: System
Instrument: Biochrom 30 (Offline)

Rep 1 Data File	
Rep 1 Sample ID	
Rep 1 Calib. Time	

	Level 12
Amount	10000
Area	1642958
RF	0.006086582
	85847843
Last Area	
Residual	5.10509
Rep StDev	
Rep %RSD	
Rep 1 Area	1642958
Rep 1 User	
Rep 1 Data File	
Rep 1 Sample ID	
Rep 1 Calib. Time	

14 Data interpretation

14.1 Limitations of the procedure

Normal range values shown earlier refer to samples taken from individuals free from PKU. Values which are below the normal range also indicate absence of PKU. All patients have values above this range and most have values at least twice the maximum range.

All data should be interpreted in the context of the following performance characteristics:

- Repeatability of analysis +/- 1.5%
- Reproducibility of samples: within +/-5%
- Linearity range 9.8 pmoles to 10 nmoles, correlation coefficient 0.9999.
- Sensitivity of analysis: detection of 15pmoles at signal/noise ratio of 3:1
- Diagnostic sensitivity: Phenylalanine levels > 71 micromoles/L
- Upper operational limit (with unlimited sample vol.): Phenylalanine 25,000 micromoles/L
- Lower operational limit: Phenylalanine, 15 nanomoles/L
- Use of sample volumes below 10microlitres, either with the objective of staying within the upper operational limit or because of limited sample availability, will result in the injection repeatability exceeding 1% RSD.

Test results are not intended to be sole diagnostic tool and must be interpreted in context with other laboratory and clinical findings.

14.2 Measurement anomalies

Anomalous analytical data may be caused by several factors (see also sample preparation and operation sections). Values which are high or otherwise suspect should therefore be followed up with the pediatrician to enable re-sampling and subsequent treatment if the measurement is validated.

Examples of factors relating to sample preparation are:

- Inadvertent addition of phenylalanine during sampling.
- Failure to remove protein.
- Loss of sample integrity due to extended storage above -20degC
- Contamination by common chemicals [For example samples have been contaminated by the presence of aspartame on the skin of the patient. This subsequently breaks down to phenylalanine during the sample preparation and gives a false positive reading].

In some circumstances it is possible to exceed the upper measuring limit of the kits when used on the Bio30+ instrument. Saturation of the detector will cause a sudden non-linearity in the quantity/response graph with a sharply lessened or horizontal slope or hook in the curve, the so- called 'high-dose hook effect'. This is easily checked by inspection of the chromatographs which will display flat-topped peaks with a y-axis response >1volt.
If the cause is deliberate injection of large quantities to observe other low-level peaks, then the issue can be ignored: the column itself is not overloaded and will continue to perform correctly.
If there is a requirement to measure the saturating peak, then steps must be taken to bring it on scale. The simplest option is to reduce the injection volume. However, caution must be taken when injecting very small volumes because the reproducibility falls along with the injection volume. We advise against using less than 10microlitres and strongly advise against approaching the absolute limit for the autosampler [1microlitre in the case of the Biochrom

Alias equipment].

A better approach is to dilute the sample and the following points will guide the process:

1. Use Loading Buffer 80-2038-10 as diluent, in preference to water, as this will stabilise the pH of the injected sample.
2. Ensure any internal standard is added to the undiluted sample so that the dilution ratio can be cross-checked between samples and also, if required, with an external reference sample.
3. Dilute the samples volumetrically, not gravimetrically, using calibrated pipetting tools and plastic tips. Use simple ratios and do not serially dilute the samples over more than 3 steps.
4. The accuracy of the dilution should be verified by calculating the linearity of the graph of peak area against quantity for the internal standard.

15 Maintenance of instrument performance

15.1 Routine service requirements

User maintenance should be carried out according to the daily, weekly and monthly checks detailed in the user manual and help pages of the Biochrom AAA software. The correct performance of the Analyser should also be maintained by scheduled service attention from Biochrom-trained personnel

15.2 Performance Qualification

Routine operation of the Analyser for satisfactory operation should be assessed by the following performance qualification

15.3 Peak Area Reproducibility

Chromatograms of Biochrom supplied standard are run (a minimum of 6 runs) and for phenylalanine peak area the %CV is calculated. %CV should be less than or equal to 1.5%.

15.4 Retention Time Reproducibility

In the same chromatograms of Biochrom standards (a minimum of 6 runs) and for phenylalanine peak area the %CV is calculated.
%CV should be less than or equal to 0.5%.

15.5 Peak Resolution

In the above chromatograms resolution of phenylalanine from other amino acids should be greater or equal to 1.5 (USP resolution calculation method).

15.6 Diagnostic guide and fault detection system

In the event of incorrect analysis the following system checks should be carried out:

1. Analyse the Biochrom supplied test mixture. If the standard is correct, then the fault could be contamination of the sample, in which case re-sample.

2. If the standard is also incorrect, the cause will be one of the following:

15.6.1 All peaks missing

Sample vial empty, autosampler not operating or operator error - no standard loaded, reaction coil switched off, photometer lamp off.

15.6.2 Extra 'ghost' peaks

Contamination of the standard mixture, of the loading buffer or of chromatography buffers.

15.6.3 Peaks too small

- Incorrect or degraded ninhydrin reagent,
- Badly diluted or degraded standard mixture,
- Dirty flow cell or a low temperature reaction coil.

15.6.4 Poor resolution

Usually due to contaminated column resin or headspace.

15.6.5 Some peaks missing on the trace

Associated with incorrect programming.

15.6.6 Poor retention time reproducibility

Buffer pump leaking, leak in buffer fluidics, air in autosampler syringe, incorrect programming.

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17 Warranty

The procedures described in these instructions are for application to the Biochrom Amino Acid Analyser and chemicals supplied by Biochrom Ltd. Any deviation from these procedures which is not recommended by Biochrom Ltd may invalidate all encompassed warranties and derived claims.

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