

F. WARNINGS AND PRECAUTIONS

1. The kit has to be used by skilled and properly trained technical personnel only, under the supervision of a medical doctor responsible of the laboratory.

2. When the kit is used for the screening of blood units and blood components, it has to be used in a laboratory certified and qualified by the national authority in that field (Ministry of Health or similar entity) to carry out this type of analysis.

3. All the personnel involved in performing the assay have to wear protective laboratory clothes, face-free gloves and glasses. The use of any sharp needles or cutting blades devices should be avoided. All the personnel involved should be trained in biosafety procedures, as recommended by the Center for Disease Control, Atlanta, U.S. and reported in the National Institute of Health's publication: "Biosafety in Microbiological and Biomedical Laboratories", ed. 1984.

4. All the personnel involved in sample handling should be vaccinated for HBV and HAV, for which vaccines are available, safe and effective.

5. The laboratory environment should be controlled so as to avoid contaminants such as dust or air-borne microbial agents, when opening kit vials and microplates and when performing the test. Protect the Chromogen (TMB) from strong light and avoid vibration of the bench surface where the test is undertaken.

6. Upon receipt, store the kit at 2-8°C into a temperature controlled refrigerator or cold room.

7. Do not interchange components between different lots of the kits. It is recommended that components between two kits of the same lot should not be interchanged.

8. Check that the reagents are clear and do not contain visible heavy particles or aggregates. If not, advise the laboratory supervisor to initiate the necessary procedures for kit replacement.

9. Avoid cross-contamination between serum/plasma samples by using disposable tips and changing them after each sample.

10. Avoid cross-contamination between kit reagents by using disposable tips and changing them between the use of each one.

11. Do not use the kit after the expiration date stated on the external container and internal (vials) labels. A study conducted on an opened kit has not pointed out any relevant loss of activity up to 6 re-use of the device and up to 6 months.

12. Treat all specimens as potentially infective. All human serum specimens should be handled at Biosafety Level 2, as recommended by the Center for Disease Control, Atlanta, U.S. in compliance with what reported in the Institutes of Health's publication: "Biosafety in Microbiological and Biomedical Laboratories", ed. 1984.

13. The use of disposable plastic-ware is recommended in the preparation of the liquid components or in transferring components into automated workstations, in order to avoid cross contamination.

14. Waste produced during the use of the kit has to be discarded in compliance with national directives and laws concerning laboratory waste of chemical and biological substances. In particular, liquid waste generated from the washing procedure, from residuals of controls and from samples has to be treated as potentially infective material and inactivated before waste. Suggested procedures of inactivation are treatment with a 10% final concentration of household bleach for 16-18 hrs or heat inactivation by autoclave at 121°C for 20 min.

15. Accidental spills from samples and operations have to be adsorbed with paper tissues soaked with household bleach and then with water. Tissues should then be discarded in proper containers designated for laboratory/hospital waste.

16. The Stop Solution is an irritant. In case of spills, wash the surface with plenty of water.

17. Other waste materials generated from the use of the kit (example: tips used for samples and controls, used microplates) should be handled as potentially infective and disposed according to national directives and laws concerning laboratory wastes.

6. Enzyme Conjugate Diluent (CONJ DIL) 2x15ml/vial. Ready to use and pink/red color coded reagent. It contains 10 mM Tris buffer pH 6.8+/-0.1, 1.1% normal mouse serum, 5% BSA, 0.1% Kathon GC and 0.02% gentamicine sulphate as preservatives. The solution is normally opalescent.

7. Enzyme Conjugate (CONJ 20X) 2x1ml/vial. 20X concentrated reagent. It contains Horseradish Peroxidase (HRP) labeled mouse monoclonal antibodies to HBsAg, determinant "a" and "pre-S", 10 mM Tris buffer pH 6.8+/-0.1, 5% BSA, 0.1% Kathon GC and 0.02% gentamicine sulphate as preservatives.

8. Chromogen/Substrate (SUBS TMB) 2x25ml/bottle. It contains a 50 mM citrate-phosphate buffered solution at pH 3.5-3.8, 4% dimethylsulphoxide, 0.03% tetra-methyl-benzidine (TMB) and 0.02% hydrogen peroxide (H2O2).

Note: To be stored protected from light as sensitive to strong illumination.

9. Sulphuric Acid (H2SO4 0.3M) 1x25ml/bottle. It contains 0.3 M H2SO4 solution.

Note: Attention: Irritant (H315; H319; P280; P302+P352; P332+P313; P305+P351+P338; P337+P313; P362+P363)

10. Plate sealing foils n° 4

Microplates		N°5		N°10	
Negative Control	1x10ml/vial	1x10ml/vial	1x20ml/vial	1x20ml/vial	1x20ml/vial
Positive Control	1x10ml/vial	1x10ml/vial	1x20ml/vial	1x20ml/vial	1x20ml/vial
Calibrator	1x10ml/vial	1x10ml/vial	1x20ml/vial	1x20ml/vial	1x20ml/vial
Wash buffer concentrate	1x60ml/vial	1x60ml/vial	1x60ml/vial	1x60ml/vial	1x60ml/vial
Enzyme conjugate	1x10ml/vial	1x10ml/vial	1x10ml/vial	1x10ml/vial	1x10ml/vial
Conjugate Diluent	1x10ml/vial	1x10ml/vial	1x10ml/vial	1x10ml/vial	1x10ml/vial
Chromogen/Substrate	1x25ml/vial	1x25ml/vial	1x25ml/vial	1x25ml/vial	1x25ml/vial
Sulphuric Acid	1x15ml/vial	1x15ml/vial	1x15ml/vial	1x15ml/vial	1x15ml/vial
Plate sealing foils	N° 1	N° 1	N° 1	N° 1	N° 1
Package insert	N° 1	N° 1	N° 1	N° 1	N° 1
Number of tests	96	480	480	960	960
Code SAGIULTRA.CE	96	480	480	960	960

E. MATERIALS REQUIRED BUT NOT PROVIDED

1. Calibrated Micropipettes (150ul, 100ul and 50ul) and disposable plastic tips.
2. EIA grade water (double distilled or deionised, charcoal treated) to remove oxidizing chemicals used as disinfectants.
3. Timer with 60 minute range or higher.
4. Absorbent paper tissues.
5. Calibrated ELISA microplate thermostatic incubator (dry or wet), capable to provide shaking at 1300 rpm+/-150, set at +37°C.
6. Calibrated ELISA microwell reader with 450nm (reading) and with 620-630nm (blanking) filters.
7. Calibrated ELISA microplate washer.
8. Vortex or similar mixing tools.

6. Enzyme conjugate:

The working solution is prepared by diluting the 20X concentrated reagent into the Conjugate Mix well on vortex before use.

Avoid any contamination of the liquid with oxidizing chemicals, dust or microbes. If this component has to be transferred, use only plastic sterile disposable containers.

Important note: The working solution is not stable. Prepare only the volume necessary for the work of the day. As an example when the kit is used in combination with other instruments or manually dilute 0.1 ml 20X Conjugate with 1.9 ml Conjugate Diluent into a disposable plastic vial and mix carefully before use.

7. Chromogen/Substrate:

Avoid contamination of the liquid with oxidizing chemicals, air-dried dust or microbes. Do not expose to strong light, oxidizing agents and metallic surfaces. If this component has to be transferred use only plastic, and if possible, sterile disposable container.

8. Sulphuric Acid:

Ready to use. Mix well by end-over-end mixing. Attention: Irritant (H315; H319; P280; P302+P352; P332+P313; P305+P351+P338; P337+P313; P362+P363).

Legend:

Warning H statements:

H315 – Causes skin irritation.

H319 – Causes serious eye irritation.

Precautinary P statements:

P280 – Wear protective gloves/protective clothing/eye protection/face protection.

P302 + P352 – IF ON SKIN: Wash with plenty of soap and water.

P332 + P313 – If skin irritation occurs: Get medical advice/attention.

P305 + P351 + P338 – IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.

P337 + P313 – If eye irritation persists: Get medical advice/attention.

P362 + P363 – Take off contaminated clothing and wash it before reuse.

I. INSTRUMENTS AND TOOLS USED IN COMBINATION WITH THE KIT

1. Micropipettes have to be calibrated to deliver the correct volume required by the assay and must be submitted to regular decontamination (70% ethanol, 10% solution of bleach, hospital grade disinfectants) of those parts that could accidentally come in contact with the sample or the components of the kit. They should also be regularly maintained in order to show a precision of 1% and a trueness of +2%.
2. The ELISA incubator has to be set at +37°C (tolerance of +1°C) and regularly checked to ensure the correct temperature is maintained. Both dry incubators and water baths are suitable for the incubations, provided that the instrument is validated for the incubation of ELISA tests.
3. In case of shaking during incubations the instrument has to ensure 350 rpm +150. Amplitude of shaking is very important as a wrong one could give origin to splashes and therefore to some false positive result.
4. The ELISA washer is extremely important to the overall performance of the assay. The washer must be carefully validated and correctly optimized using the kit controls/calibrator and reference panels before using them for routine laboratory tests. Usually 4-5 washing cycles (aspiration + dispensation of 350ul/well of washing solution = 1 cycle) are sufficient to ensure that the assay performs as expected. A soaking time of 20-30 seconds between cycles is suggested. In order to set correctly their number,

is recommended to run an assay with the kit controls/calibrator and well-characterized negative and positive reference samples, and check to match the values reported below in the section "Internal Quality Control". Regular calibration of the volumes delivered and maintenance (decontamination and cleaning of needles) of the washer has to be carried out according to the instructions of the manufacturer.

5. Incubation times have a tolerance of $\pm 5\%$.

6. The microplate reader has to be equipped with a reading filter of 450nm and with a second filter (620-630nm; strongly recommended) for blanking purposes. Its standard performances should be: (a) bandwidth ≤ 10 nm; (b) absorbance range from 0 to ≥ 2.0 ; (c) linearity to ≥ 2.0 ; (d) repeatability $\geq 1\%$. Blanking is carried out on the well identified in the section "Assay Procedure". The optical system of the reader has to be calibrated regularly to ensure that the correct optical density is measured. It should be regularly maintained according to the manufacturer's instructions.

7. When using ELISA automated workstations, all critical steps (dispensation, incubation, washing, reading, shaking, data handling, etc.) have to be carefully set, calibrated, controlled and regularly serviced in order to match the values reported in the sections "Internal Quality Control". The assay protocol has to be installed in the operating system of the unit and validated by checking full matching the declared performances of the kit. In addition, the liquid handling part of the station (dispensation and washing) has to be validated and correctly set paying particular attention to avoid carry over by the needles used for dispensing samples and for washing. The carry over effect must be studied and controlled to minimize the possibility of contamination of adjacent wells due to strongly reactive samples, leading to false positive results. The use of ELISA automated work stations is recommended for blood screening and when the number of samples to be tested exceed 20-30 units per run.

8. When using automatic devices, in case the vial holder of the instrument does not fit with the vials supplied in the kit, transfer the solution into appropriate containers and label them with the same label peeled out from the original vial. This operation is important in order to avoid mismatching contents of vials, when transferring them. When the test is over, return the secondary labeled containers to 2-8°C, firmly capped.

9. Dia.Pro's customer service offers support to the user in the setting and checking of instruments used in combination with the kit, in order to assure full compliance with the essential requirements of the assay. Support is also provided for the installation of new instruments to be used in combination with the kit.

L. PRE ASSAY CONTROLS AND OPERATIONS

1. Check the expiration date of the kit printed on the external label of the kit box. Do not use if expired.
2. Check that the liquid components are not contaminated by naked-eye visible particles or aggregates. Check that Chromogen/Substrate is colorless or pale blue. Check that no leakage occurred in transportation and no spillage of liquid is present inside the box. Check that the aluminum pouch, containing the microplate, is not punctured or damaged.
3. Dilute all the content of the 20x concentrated Wash Solution as described above.
4. Dilute the 20x concentrated Enzyme Conjugate with its Diluent as reported.
5. Dissolve the Calibrator as described above.
6. Allow all the other components to reach room temperature (about 1 hr) and then mix as described.
7. Set the ELISA incubator at $+37^{\circ}\text{C}$ and prepare the ELISA washer by priming with the diluted washing solution.

according to the manufacturers instructions. Set the right number of washing cycles as found in the validation of the instrument for its use with the kit.

8. Check that the ELISA reader has been turned on at least 20 minutes before reading.

9. If using an automated workstation, turn it on, check settings and be sure to use the right assay protocol.

10. Check that the micropipettes are set to the required volume.

11. Check that "all the other equipment" is available and ready to use.

12. In case of problems, do not proceed further with the test and advise the supervisor.

M. ASSAY PROCEDURE

The assay has to be carried out according to what reported below, taking care to maintain the same incubation time for all the samples in testing.

Automated assay:

In case the test is carried out automatically with an ELISA system, we suggest to make the instrument dispense first 150 μl controls & calibrator, then all the samples and finally 100 μl diluted Enzyme Conjugate.

For the pre-washing step (point 1 of the assay procedure) and all the next operations follow the operative instructions reported below for the Manual Assay.

It is strongly recommended to check that the time lap between the dispensation of the first and the last sample will be calculated by the instrument and taken into consideration by delaying the first washing operation accordingly.

Manual Assay:

1. Place the required number of strips in the plastic holder and wash them once to hydrate wells. Carefully identify the wells for controls, calibrator and samples.

Important note: Pre washing (1 cycle: dispensation of 350 μl of washing solution+ aspiration) is fundamental to obtain reliable and specific results both in the manual and in the automatic procedures. Do not omit it!

2. Leave the A1 well empty for blanking purposes.
3. Pipette 150 μl of the Negative Control in triplicate, 150 μl of the Calibrator in duplicate and then 150 μl of the Positives. Control in single followed by 150 μl of each of the samples.
4. Check for the presence of samples in wells by naked eye (there is a marked color difference between empty and full wells) or by reading at 450/620nm. (samples show OD values higher than 0.100).
5. Dispense 160 μl diluted Enzymatic Conjugate in all wells, except for A1, used for blanking operations.

Important note: Be careful not to touch the inner surface of the well with the pipette tip when the conjugate is dispensed. Contamination might occur.

6. Following addition of the conjugate, check that the color of the samples have changed from yellowish to pink/red and then incubate the microplate for 120 min at $+37^{\circ}\text{C}$.

Important notes:

- a. Strips have to be sealed with the adhesive sealing foil, only when the test is performed manually. Do not cover strips when using ELISA automatic instruments.
 - b. If the procedure is carried out on shaking, be sure to deliver the rpm reported for in Section I.3 as otherwise intra-well contamination could occur.
7. When the first incubation is over, wash the microwells as previously described (section I.4)

8. Pipette 200 μl Chromogen/Substrate into all the wells. A1 section:

Microplate

	1	2	3	4	5	6	7	8	9	10	11	12
A	BLK	S2										
B	NC	S3										
C	NC	S4										
D	NC	S5										
E	CAL	S6										
F	CAL	S7										
G	PC	S8										
H	S1	S9										

Legend: BLK = Blank NC = Negative Control
CAL = Calibrator PC = Positive Control S = Sample

Important general notes:

1. If the second filter is not available, ensure that no fingerprints or dust are present on the external bottom of the microwell before reading at 450nm. They could generate false positive results on reading.
2. Reading should ideally be performed immediately after the addition of the acid solution but definitely no longer than 20 minutes afterwards. Some self-oxidation of the chromogen can occur leading to a higher background.
3. When samples to be tested are not surely clean or have been stored frozen the assay procedure reported below is recommended as long as it is far less sensitive to interferences due to hemolysis, hyperlipaemia, bacterial contamination and fibrin microprecipitates. The assay is carried out in two-steps at $+37^{\circ}\text{C}$ on shaking at 350 rpm ± 150 as follows:
 - dispense 100 μl of controls, calibrator and samples incubate 60 min at $+37^{\circ}\text{C}$ on shaking
 - wash according to instructions (section I.4)
 - dispense 100 μl diluted enzyme tracer
 - incubate 30 min at $+37^{\circ}\text{C}$ on shaking
 - wash
 - dispense 100 μl TMB/H2O2 mix
 - incubate 30 min at r.t. on shaking
 - stop and readIn this procedure the pre-wash can be omitted. This method shows performances similar to the standard one and therefore can be used in alternative.
4. The Calibrator (CAL) does not affect the cut-off calculation and therefore the test results calculation. The Calibrator may be used only when a laboratory internal quality control is required by the management.

O. INTERNAL QUALITY CONTROL

A check is performed on the controls/calibrator any time the kit is used in order to verify whether the expected OD450nm or S/Co values have been matched in the analysis.

Ensure that the following results are met:

Parameter	Requirements
Blank well	< 0.100 OD450nm value
Negative Control (NC)	< 0.050 mean OD450nm value after blanking
Calibrator 0.5 IU/ml	S/Co > 2
Positive Control	> 1.000 OD450nm value

If the results of the test match the requirements stated above, proceed to the next section.
If they do not, do not proceed any further and perform the following checks:

Problem	Check
Blank well > 0.100 OD450nm	1. that the Chromogen/Substrate solution has not become contaminated during the assay
Negative Control (NC) > 0.050 OD450nm after blanking	1. that the washing procedure and the washer settings are as validated in the pre qualification study; 2. that the proper washing solution has been used and the washer has been primed with it before use; 3. that no mistake has been done in the assay procedure (dispensation of positive control instead of the negative one); 4. that no contamination of the negative control or of the wells where the control was dispensed has occurred due to spills of positive samples or of the enzyme conjugate; 5. that micropipettes have not become contaminated with positive samples or with the enzyme conjugate 6. that the washer needles are not blocked or partially obstructed.

N. ASSAY SCHEME

Operations	Procedure
Pre-Washing step	n° 1 cycle
Controls&Calibrator&samples	150 μl
Diluted Enzyme Conjugate	100 μl
1 st incubation	120 min
Temperature	$+37^{\circ}\text{C}$
Washing steps	n° 4-5
Chromogen/Substrate	200 μl
2 nd incubation	30 min
Temperature	room
Suphanic Acid	100 μl
Reading OD	450nm

Calibrator S/Co < 2	1. that the procedure has been correctly performed;
	2. that no mistake has occurred during its distribution (ex: dispensation of negative control instead of calibrator)
Positive Control < 1.000 OD450nm	3. that the washing procedure and the washer settings are as validated in the pre qualification study;
	4. that no external contamination of the calibrator has occurred.
Positive Control < 1.000 OD450nm	1. that the procedure has been correctly performed;
	2. that no mistake has occurred during the distribution of the control (dispensation of negative control instead of positive control. In this case, the negative control will have an OD450nm value > 0.050).
Positive Control < 1.000 OD450nm	3. that the washing procedure and the washer settings are as validated in the pre qualification study;
	4. that no external contamination of the positive control has occurred.

If any of the above problems have occurred, report the problem to the supervisor for further actions.

P. CALCULATION OF THE CUT-OFF
The test results are calculated by means of a cut-off value determined on the mean OD450nm value of the negative control (NC) with the following formula:

$$NC + 0.050 = \text{Cut-Off (Co)}$$

The value found for the test is used for the interpretation of results as described in the next paragraph.

Important note: When the calculation of results is performed by the operating system of an ELISA automated work station, ensure that the proper formulation is used to calculate the cut-off value and generate the correct interpretation of results.

Q. INTERPRETATION OF RESULTS
Test results are interpreted as a ratio of the sample OD450nm (S) and the Cut-Off value (Co), mathematically S/Co, according to the following table:

S/Co	Interpretation
< 0.9	Negative
0.9 – 1.1	Equivocal
> 1.1	Positive

A negative result indicates that the patient is not infected by HBV and that the blood unit may be transfused.
Any patient showing an equivocal result should be retested on a second sample taken 1-2 weeks after the initial sample, the blood unit should not be transfused.
A positive result is indicative of HBV infection and therefore the patient should be treated accordingly or the blood unit should be discarded.

Important notes:
1. Interpretation of results should be done under the supervision of the laboratory supervisor to reduce the risk of judgment errors and misinterpretations.
2. Any positive result must be confirmed first by repeating the test on the sample, after having filtered it on 0.2-0.8 µ filter to remove any microparticles interference. Then, if still

positive, the sample has to be submitted to a confirmation test before a diagnosis of viral hepatitis is released.
3. When test results are transmitted from the laboratory to another department, attention must be paid to avoid erroneous data transfer.
4. Diagnosis of viral hepatitis infection has to be taken and released to the patient by a suitably qualified medical doctor.

An example of calculation is reported below.

The following data must not be used instead of real figures obtained by the user.
Negative Control: 0.012 – 0.008 – 0.010 OD450nm
Mean Value: 0.010 OD450nm
Lower than 0.050 – Accepted
Positive Control: 2.489 OD450nm
Higher than 1.000 – Accepted
Cut-Off = 0.010 + 0.050 = 0.060
Calibrator: 0.350 – 0.370 OD450nm S/Co = 6.0
Mean value: 0.360 OD450nm
S/Co higher than 2.0 – Accepted
Sample 1: 0.028 OD450nm
Sample 2: 1.690 OD450nm
Sample 1 S/Co < 0.9 = negative
Sample 2 S/Co > 1.1 = positive

R. PERFORMANCE CHARACTERISTICS
Evaluation of Performances has been conducted in accordance to what reported in the Common Technical Specifications or CTS (art. 5, Chapter 3 of IVD Directive 98/79/EC). Version ULTRA proved to be at least equivalent to the original design in a study conducted for the validation of the new version.

1. Analytical Sensitivity
The limit of detection of the assay has been calculated on the 2nd WHO international standard, NIBSC code 00588.
In the following table, results are given for three lots (P1, P2 and P3) of the version ULTRA in comparison with the reference device (Ref.):

WHO IU/ml	Lot # P1 S/Co	Lot # P2 S/Co	Lot # P3 S/Co	Ref. S/Co
0.4	4.6	4.8	4.6	4.6
0.2	2.3	2.4	2.4	2.4
0.1	1.4	1.4	1.5	1.2
0.05	0.8	0.8	1.0	0.7
FCS (NC)	0.3	0.2	0.3	0.1

The assay shows an Analytical Sensitivity better than 0.1 WHO IU/ml of HBsAg.

In addition two panels of sensitivity supplied by EFS, France, and by SFTS, France, were tested and gave in the best conditions the following results:

Panel EFS Ag HBs HB1-HB6 lot n° 04

Sample ID	Characteristics	ng/ml	S/Co
HB1	diluent	/	0.2
HB2	adw2+ayw3	0.05	0.6
HB3	adw2+ayw3	0.1	1.0
HB4	adw2+ayw3	0.2	1.8
HB5	adw2+ayw3	0.3	2.4
HB6	adw2+ayw3	0.5	4.2

Sensitivity panel SFTS, France, Ag HBs 2005

Sample ID	Characteristics	ng/ml	S/Co
171	Adw2+ayw3	2.21 ± 0.15	15.4
172	Adw2+ayw3	1.18 ± 0.10	8.7
173	Adw2+ayw3	1.02 ± 0.05	6.1
174	Adw2+ayw3	0.64 ± 0.04	4.0
175	Adw2+ayw3	0.49 ± 0.03	3.4
176	Adw2+ayw3	0.39 ± 0.02	2.6
177	Adw2+ayw3	0.25 ± 0.02	2.0
178	Adw2+ayw3	0.11 ± 0.02	1.3
179	Adw2+ayw3	0.06 ± 0.01	0.9
180	Adw2+ayw3	0.03 ± 0.01	0.6
181	Adw2	0.5 – 1.0	4.7
182	Adw2	0.5 – 1.0	3.6
183	Adw2	0.5 – 1.0	4.5
184	Adw2	0.5 – 1.0	5.1
185	Adw2	0.5 – 1.0	6.4
186	Adw2	0.5 – 1.0	7.3
187	Adw2	0.5 – 1.0	5.8
188	Adw2	0.5 – 1.0	6.9
189	Adw2	0.5 – 1.0	6.1
190	diluent	/	0.6

The panel # 808, supplied by Boston Biomedical Inc., USA, was also tested to define the limit of sensitivity.
Results in the best conditions are as follows:

BBi panel PHA 808

Sample ID	Characteristics	ng/ml	S/Co
01	ad	2.49	10.2
02	ad	1.17	4.8
03	ad	1.02	4.3
04	ad	0.96	3.8
05	ad	0.89	2.9
06	ad	0.90	2.2
07	ad	0.41	1.5
08	ad	0.37	1.3
09	ad	0.30	1.2
10	ad	0.23	1.0
11	ay	2.51	11.2
12	ay	1.26	5.9
13	ay	0.97	4.1
14	ay	0.77	3.7
15	ay	0.63	2.9
16	ay	0.48	2.0
17	ay	0.42	1.8
18	ay	0.33	1.6
19	ay	0.32	1.6
20	ay	0.13	1.1
21	negative	/	0.6

2. Diagnostic Sensitivity:
The diagnostic sensitivity was tested according to what required by Common Technical Specifications (CTS) of the directive 98/79/EC on IVD for HBsAg testing.
Positive samples, including HBsAg subtypes and a panel of "s" mutants for most frequent mutations, were collected from different HBV pathologies (acute, asymptomatic and chronic hepatitis B) or produced synthetically, and were detected positive in the assay.
All the HBsAg known subtypes, "ay" and "ad", and isoforms "w" and "r", supplied by CTS, France, were tested in the assay and determined positive by the kit as expected.
An overall value of 100% has been found in a study conducted on a total number of more than 400 samples positive with the original reference IVD code SAG1.CE, CE marked.
A total of 30 sero-conversions were studied, most of them produced by Boston Biomedical Inc., USA.

Results obtained by examining eight panels supplied by Boston Biomedical Inc., USA, are reported below for the version ULTRA in comparison with the reference device code SAG1.CE.

Panel ID	1 st sample	HBsAg subtype	HBsAg ng/ml	Version ULTRA S/Co	Ref. device S/Co
PHM 906	02	ad	0.5	3.7	1.4
PHM 907 (M)	06	ay	1.0	4.4	2.9
PHM 909	04	ad	0.3	1.2	0.8
PHM 914	04	ad	0.5	1.1	1.1
PHM 946	02	ad	0.4	1.6	0.6
PHM 923	03	ay	< 0.2	2.2	1.2
PHM 925	03	ad	n.d.	1.4	0.9
PHM 934	01	ad	n.d.	1.0	0.8

3. Diagnostic Specificity:
It is defined as the probability of the assay of scoring negative in the absence of specific analyte. In addition to the first study, where more than 5000 negative samples from blood donors (two blood centers), classified negative with a CE marked device in use at the laboratory of collection were examined, the diagnostic specificity was recently assessed by testing a total of 2288 negative blood donors on seven different lots. A value of specificity of 100% was found.
Both plasma, derived with different standard techniques of preparation (citrate, EDTA and heparin), and sera have been used to determine the specificity.
No false reactivity due to the method of specimen preparation has been observed.
Frozen specimens have also been tested to check whether samples freezing interferes with the performance of the test.
No interference was observed on clean and particle free samples.
Samples derived from patients with different viral (HCV, HAV) and non viral pathologies of the liver that may interfere with the test were examined. No cross reaction were observed.

4. Precision:
It has been calculated for the version ULTRA on two samples examined in 16 replicates in 3 different runs for three lots. Results are reported in the following tables:

Average values	Negative Sample	Calibrator
Total n = 144		0.5 IU/ml
OD450nm	0.026	0.332
Std.Deviation	0.004	0.027
CV %	16%	8%

The variability shown in the tables did not result in sample misclassification.

S. LIMITATIONS
Repeatable false positive results were assessed on freshly collected specimens in less than 0.1% of the normal population, mostly due to high titers Heterophilic Anti Mouse Antibodies (HAMA).
Interferences in fresh samples were also observed when they were not particles-free or were badly collected (see chapter G).
Old or frozen samples, presenting fibrin clots, cryoglobulins, lipid-containing micelles or microparticles after storage or thawing, can generate false positive results.

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All the IVD Products manufactured by the company are under the control of a certified Quality Management System approved by an EC Notified Body. Each lot is submitted to a quality control and released into the market only if conforming with the EC technical specifications and acceptance criteria.

Manufacturer:
Dia.Pro Diagnostic Bioprobes S.r.l.
Via G. Carducci n° 27 – Sesto San Giovanni (MI) – Italy

HBsAb

Enzyme Immunoassay for qualitative/quantitative determination of antibodies to Hepatitis B surface Antigen in human serum and plasma

- for "in vitro" diagnostic use only -



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REF.SAB,CE
 96 Tests

HBs Ab

A. INTENDED USE

Enzyme Immunoassay (ELISA) for both the quantitative and qualitative determination of antibodies to the Surface Antigen of Hepatitis B Virus in human plasma and sera.
 For "in vitro" diagnostic use only.

B. INTRODUCTION

The World Health Organization (WHO) defines Hepatitis B Virus infection as follows:

"Hepatitis B is one of the major diseases of mankind and is a serious global public health problem. Hepatitis means inflammation of the liver, and the most common cause is infection with one of 5 viruses, called hepatitis A,B,C,D, and E. All of these viruses can cause an acute disease with symptoms lasting several weeks including yellowing of the skin and eyes (jaundice); dark urine; extreme fatigue; nausea, vomiting and abdominal pain. It can take several months to a year to feel fit again. Hepatitis B virus can cause chronic infection in which the patient never gets rid of the virus and many years later develops cirrhosis of the liver or liver cancer."

HBV is the most serious type of viral hepatitis and the only type causing chronic hepatitis for which a vaccine is available. Hepatitis B virus is transmitted by contact with blood or body fluids of an infected person in the same way as human immunodeficiency virus (HIV), the virus that causes AIDS. However, HBV is 50 to 100 times more infectious than HIV. The main ways of getting infected with HBV are: (a) perinatal (from mother to baby at the birth); (b) child-to-child transmission; (c) unsafe injections and transfusions; (d) sexual contact.

Worldwide, most infections occur from infected mother to child, from child to child contact in household settings, and from reuse of unsterilized needles and syringes. In many developing countries, almost all children become infected with the virus. In many industrialized countries (e.g. Western Europe and North America), the pattern of transmission is different. In these countries, mother-to-infant and child-to-child transmission accounted for up to one third of chronic infections before childhood hepatitis B vaccination programmes were implemented. However, the majority of infections in these countries are acquired during young adulthood by sexual activity, and injecting drug use. In addition, hepatitis B virus is the major infectious occupational hazard of health workers, and most health care workers have received hepatitis B vaccine.

Hepatitis B virus is not spread by contaminated food or water, and cannot be spread casually in the workplace. High rates of chronic HBV infection are also found in the southern parts of Eastern and Central Europe. In the Middle East and Indian subcontinent, about 5% are chronically infected. Infection is less common in Western Europe and North America, where less than 1% are chronically infected.

Young children who become infected with HBV are the most likely to develop chronic infection. About 90% of infants infected during the first year of life and 30% to 50% of children infected between 1 to 4 years of age develop chronic infection. The risk of death from HBV-related liver cancer or

cirrhosis is approximately 25% for persons who become chronically infected during childhood.

Chronic hepatitis B in some patients is treated with drugs called *interferon* or *lamivudine*, which can help some patients. Patients with cirrhosis are sometimes given liver transplants, with varying success. It is preferable to prevent this disease with vaccine than to try and cure it.

Hepatitis B vaccine has an outstanding record of safety and effectiveness. Since 1982, over one billion doses of hepatitis B vaccine have been used worldwide. The vaccine is given as a series of three intramuscular doses. Studies have shown that the vaccine is 95% effective in preventing children and adults from developing chronic infection if they have not yet been infected. In many countries where 8% to 15% of children used to become chronically infected with HBV, the rate of chronic infection has been reduced to less than 1% in immunized groups of children. Since 1991, WHO has called for all countries to add hepatitis B vaccine into their national immunization programmes."

Hepatitis B surface Antigen (HBsAg) is the major structural polypeptide of the envelope of the Hepatitis B virus (HBV). This antigen is composed mainly of the type common determinant "a", and the type specific determinants "d" and "y". present only on the specific serotypes.

Upon infection, a strong immunological response develops firstly against the type specific determinants and in a second time against the "a" determinant.

Anti-"a" antibodies are however recognised to be most effective in the neutralisation of the virus, protecting the patient from other infections and leading it to convalescence.

The detection of HBsAb has become important for the follow up of patients infected by HBV and the monitoring of recipients upon vaccination with synthetic and natural HBsAg.

C. PRINCIPLE OF THE TEST

Microplates are coated with a preparation of highly purified HBsAg that in the first incubation with sample specifically captures and HBsAg antibodies to the solid phase.

After washing, captured antibodies are detected by an HBsAg, labelled with peroxidase (HRP), that specifically binds the second available binding site of these antibodies.

The enzyme specifically bound to wells, by acting on the substrate/chromogen mixture, generates an optical signal that is proportional to the amount of HBsAb in the sample and can be detected by an ELISA reader.

The amount of antibodies may be quantitated by means of a standard curve calibrated against the WHO reference preparation.

Samples are pre-treated in the well with an specimen diluent able to block interference present in vaccinated individuals.

D. COMPONENTS

Each kit contains sufficient reagents to perform 96 tests.

1. Microplate: MICROPLATE

8x12 microwell strips coated with purified heat-inactivated HBsAg of both subtypes (ad and ay) from human origin and sealed into a bag with desiccant.
 Allow the microplate to reach room temperature before opening; reseal unused strips in the bag with desiccant and store at 4°C.

2. Calibration Curve: **CAL N° 1**
5x2.0 mlVial. Ready to use and colour coded standard curve, derived from HBsAg positive plasma titrated on WHO standard for anti HBsAg (1st reference preparation - 1977, lot 17-2-77), ranging: CAL1 = 10 mIU/ml // CAL2 = 10 mIU/ml // CAL3 = 50 mIU/ml // CAL4 = 100 mIU/ml // CAL5 = 250 mIU/ml.
Contains human serum proteins, 5% BSA, 10 mM phosphate buffer pH 7.4 +/- 0.1, 0.09% sodium azide and 0.1% Kathon GC as preservatives. Standards are blue coloured.

3. Wash buffer concentrate: **WASH-BUF-20X**
1x60ml bottle. 20x concentrated solution.
Once diluted, the wash solution contains 10 mM phosphate buffer pH 7.0 +/- 0.2, 0.05% Tween 20 and 0.1% Kathon GC.

4. Enzyme conjugate: **CONJ**
1x15.0 mlVial. Ready-to-use solution and red color coded. It contains inactivated purified HBsAg of both subtypes ad and ay, labelled with HRP. 5% BSA, 10 mM Tris buffer pH 6.8 +/- 0.1, 0.3 mg/ml gentamicine sulphate and 0.1% Kathon GC as preservatives.

5. Chromogen/Substrate: **SUBS-TMB**
1x16mlVial. Contains a 50 mM citrate-phosphate buffered solution at pH 3.5-3.8, 4% dimethylsulphoxide, 0.03% tetra-methyl-benzidine (TMB) and 0.02% hydrogen peroxide (H₂O₂).
Note: To be stored protected from light as sensitive to strong illumination.

6. Sulphuric Acid: **H2SO4 0.3M**
1x15mlVial. Contains 0.3 M H₂SO₄ solution.
Attention: Irritant (H315, H319; P280; P302+P352, P332+P313; P305+P351+P338, P337+P313, P362+P363).

7. Specimen Diluent: **DILSEP**
1x8ml. 10 mM Tris Buffered solution pH 7.4 +/- 0.1, suggested to be used in the follow up of vaccination. It contains 0.09% sodium azide as preservatives.

8. Control Serum: **CONTROL .m**
1 vial. Lyophilized.
Contains fetal bovine serum proteins, human anti HBsAg antibodies calibrated at 50 ± 10% WHO mIU/ml. 0.3 mg/ml gentamicine sulphate and 0.1% Kathon GC as preservatives.

9. Plate sealing foil n° 2

10. Package insert n° 1

E. MATERIALS REQUIRED BUT NOT PROVIDED

1. Calibrated Micropipettes (100ul and 50ul) and disposable plastic tips.
2. EIA grade water (double distilled or deionised), charcoal treated to remove oxidizing chemicals used as disinfectants).
3. Timer with 60 minute range or higher.
4. Absorbent paper tissues.
5. Calibrated ELISA microplate thermostatic incubator (dry or wet, set at +37°C (+/-1°C tolerance)).
6. Calibrated ELISA microwell reader with 450nm (reading) and with 620-630nm (blanking, strongly recommended) filters.
7. Calibrated ELISA microplate washer.
8. Vortex or similar mixing tools.

F. WARNINGS AND PRECAUTIONS

1. The kit has to be used by skilled and properly trained technical personnel only, under the supervision of a medical doctor responsible of the laboratory.

2. Samples have to be clearly identified with codes or names in order to avoid misinterpretation of results. Bar code labeling and electronic reading is strongly recommended.

3. Haemolyzed ("red") and visibly hyperlipemic ("milky") samples have to be discarded as they could generate false results. Samples containing residues of fibrin or heavy particles or microbial filaments and bodies should be discarded as they could give rise to false results.

4. Sera and plasma can be stored at -2°...-8°C for up to five days after collection. For longer storage periods, samples can be stored frozen at -20°C for several months. Any frozen samples should not be freeze/thawed more than once as this may generate particles that could affect the test result.
5. If particles are present, centrifuge at 2000 rpm for 20 min or filter using 0.2-0.8u filters to clean up the sample for testing.
6. Samples whose anti-HBsAg antibody concentration is expected to be higher than 250 mIU/ml should be diluted before use either 1:10 or 1:100 in the Calibrator 0 mIU/ml. Dilutions have to be done in clean disposable tubes by diluting 50 ul of each specimen with 450 ul of Cal 0 (1:10). Then 50 ul of the 1:10 dilution are diluted with 450 ul of the Cal 0 (1:100). Mix tubes thoroughly on vortex when preparing the diluted samples.

H. PREPARATION OF COMPONENTS AND WARNINGS

1. Microplate:
Allow the microplate to reach room temperature (about 1 hr) before opening the container. Check that the desiccant has not turned green, indicating a defect in conservation.

In this case, call Dia.Pro's customer service.
Unused strips have to be placed back into the aluminum pouch, with the desiccant supplied, firmly zipped and stored at +2°-8°C. After first opening, remaining strips are stable until the humidity indicator inside the desiccant bag turns from yellow to green.

2. Calibration Curve

Ready to use. Mix well on vortex before use.

3. Control Serum

Add the volume of ELISA grade water, reported on the label, to the lyophilised powder, let fully dissolve and then gently mix on vortex.
Note: The control after dissolution is not stable. Store frozen in aliquots at -20°C.

4. Wash buffer concentrate:

The whole content of the concentrated solution has to be diluted 20x with bidistilled water and mixed gently end-over-end before use. During preparation avoid foaming as the presence of bubbles could impact on the efficiency of the washing cycles.
Note: Once diluted, the wash solution is stable for 1 week at +2...8° C.

5. Enzyme conjugate:

Ready to use. Mix well on vortex before use.
Avoid contamination of the liquid with oxidising chemicals, dust or microbes. If this component has to be transferred, use only plastic, and if possible, sterile disposable containers.

6. Specimen Diluent:

Ready to use. Mix well on vortex before use.

7. Chromogen/Substrate:

Ready to use. Mix well on vortex before use.
Avoid contamination of the liquid with oxidising chemicals, air driven dust or microbes. Do not expose to strong light, oxidising agents and metallic surfaces.
If this component has to be transferred use only plastic and if possible, sterile disposable container.

8. Sulphuric Acid:

Ready to use. Mix well on vortex before use.

Attention: Irritant (H315, H319; P280, P302+P352, P332+P313, P305+P351+P338, P337+P313, P362+P363).

Legenda:

Warning H statements:

H315 – Causes skin irritation.
H319 – Causes serious eye irritation.

Precautionary P statements:

P280 – Wear protective gloves/protective clothing/eye protection/face protection.
P302 + P352 – IF ON SKIN: Wash with plenty of soap and water.
P332 + P313 – If skin irritation occurs: Get medical advice/attention.
P305 + P351 + P338 – IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
P337 + P313 – If eye irritation persists: Get medical advice/attention.
P362 + P363 – Take off contaminated clothing and wash it before reuse.

I. INSTRUMENTS AND TOOLS USED IN COMBINATION WITH THE KIT

1. Micropipettes have to be calibrated to deliver the correct volume required by the assay and must be submitted to regular decontamination (70% ethanol, 10% solution of bleach, hospital grade disinfectants) of those parts that could accidentally come in contact with the sample or the components of the kit. They should also be regularly maintained in order to show a precision of 1% and a trueness of ±2%.

2. The ELISA incubator has to be set at +37°C (tolerance of +1°C) and regularly checked to ensure the correct temperature is maintained. Both dry incubators and water baths are suitable for the incubations, provided that the instrument is validated for the incubation of ELISA tests.

3. The ELISA washer is extremely important to the overall performances of the assay. The washer must be carefully validated and correctly optimized using the kit control/calibrator and reference panels, before using the kit for routine laboratory tests. Usually 4-5 washing cycles (aspiration + dispensation of 350ul/well of washing solution = 1 cycle) are sufficient to ensure that the assay performs as expected. A soaking time of 20-30 seconds between cycles is suggested. In order to set correctly their number, it is recommended to run an assay with the kit controls/calibrator and well-characterized negative and positive reference samples, and check to match the values reported below in the sections "Validation of Test" and "Assay Performances". Regular calibration of the volumes delivered and maintenance (decontamination and cleaning of needles) of the washer has to be carried out according to the instructions of the manufacturer.

4. Incubation times have a tolerance of ±5%.
5. The ELISA microplate reader has to be equipped with a reading filter of 450nm and with a second filter (620-630nm, strongly recommended) for blanking purposes. Its standard performances should be (a) bandwidth ≤ 10 nm; (b) absorbance range from 0 to ≥ 2.0; (c) linearity to ≥ 2.0; repeatability ≥ 1%. Blanking is carried out on the well identified in the section "Assay Procedure". The optical system of the reader has to be calibrated regularly to ensure that the correct optical density is measured. It should be regularly maintained according to the manufacturer's instructions.

6. When using an ELISA automated workstation, all critical steps (dispensation, incubation, washing, reading, blanking, data handling) have to be carefully set, calibrated, controlled and regularly serviced in order to match the

values reported in the sections "Validation of Test" and "Assay Performance". The assay protocol has to be installed in the operating system of the unit and validated as for the washer and the reader. In addition, the liquid handling part of the station (dispensation and washing) has to be validated and correctly set. Particular attention must be paid to avoid carry over by the needles used for dispensing samples and for washing. This must be studied and controlled to minimize the possibility of contamination of adjacent wells due to strongly reactive samples, leading to false positive results. The use of ELISA-automated work stations is recommended for blood screening and when the number of samples to be tested exceed 20-30 units per run. Dia.Pro's customer service offers support to the user in the setting and checking of instruments used in combination with the kit, in order to assure full compliance with the requirements described. Support is also provided for the installation of new instruments to be used with the kit.

L. PRE ASSAY CONTROLS AND OPERATIONS

1. Check the expiration date of the kit printed on the external label of the kit box. Do not use if expired.
2. Check that the liquid components are not contaminated by naked-eye visible particles or aggregates. Check that the Chromogen/Substrate is colorless or pale blue by aspirating a small volume of it with a sterile transparent plastic pipette. Check that no leakage occurred in transportation and no spillage of liquid is present inside the box. Check that the aluminum pouch, containing the microplate, is not punctured or damaged.
3. Dilute all the content of the 20x concentrated Wash Solution as described above.
4. Dissolve the Control Serum as described above.
5. Allow all the other components to reach room temperature (about 1 hr) and then mix as described.
6. Set the ELISA incubator at +37°C and prepare the ELISA washer by priming with the diluted washing solution, according to the manufacturers instructions. Set the right number of washing cycles as found in the validation of the instrument for its use with the kit.
7. Check that the ELISA reader has been turned on at least 20 minutes before reading.
8. If using an automated workstation, turn it on, check settings and be sure to use the right assay protocol.
9. Check that the microplates are set to the required volume.
10. Check that all the other equipments are available and ready to use.

In case of problems, do not proceed further with the test and advise the supervisor.

M. ASSAY PROCEDURE

The assay has to be carried out according to what reported below, taking care to maintain the same incubation time for all the samples in testing.

Two procedures can be carried out with the device according to the request of the clinician.

M.1 Quantitative analysis

1. Place the required number of strips in the microplate holder. Leave A1 and B1 wells empty for the operation of blanking. Store the other strips into the bag in presence of the desiccant at 2-8°C, sealed.

Then Dispense in all the wells to be used for the test, except for A1 and B1, 50µl of the Specimen Diluent.

Important note: This additive is added before distributing samples and controls into specific wells and is particularly

intended for blocking some substances present in people undergoing vaccination and capable to mask antibodies.

2. Pipette 100µl of all the Calibrators, 100µl of Control Serum, in duplicate and then 100µl of samples. The Control Serum is used to verify that the whole analytical system works as expected. Check that Calibrators, Control Serum and samples have been correctly added. Then incubate the microplate at +37°C for 60 min.

Important note: Strips have to be sealed with the adhesive sealing foil only when the test is performed manually. Do not cover strips when using ELISA automatic instruments.

3. Wash the microplate as reported in section I.3.
4. In all the wells except A1 and B1, pipette 100 µl Enzyme Conjugate. Check that the reagent has been correctly added. Incubate the microplate at +37°C for 60 minutes.

Important note:

- 1) Be careful not to touch the inner surface of the well with the pipette tip when dispensing the Enzyme Conjugate. Contamination might occur.
- 2) Mix thoroughly the Enzyme Conjugate on vortex before use.

5. Wash the microplate as described.
6. Pipette 100µl TMB/H₂O₂ mixture in each well, the blank wells included. Check that the reagent has been correctly added. Then incubate the microplate at room temperature for 20 minutes.

Important note: Do not expose to strong direct light as a high background might be generated.

7. Stop the enzymatic reaction by pipette 100µl Sulphuric Acid into each well and using the same pipetting sequence as in step 6. Then measure the colour intensity with a microplate reader at 450nm (reading) and at 620-630nm (blanking, strongly recommended), blanking the instrument on A1 and B1 wells.

M.2 Qualitative analysis

1. Place the required number of strips in the microplate holder. Leave A1 well empty for the operation of blanking. Store the other strips into the bag in presence of the desiccant at 2-8°C, sealed.
2. Dispense 50 µl Specimen Diluent in all the wells, except for the blank A1. Then pipette 100µl of the Calibrator 0 mIU/ml in duplicate, 100µl of the Calibrator 10 mIU/ml in duplicate, 100µl of the Calibrator 250 mIU/ml in single, and then 100µl of samples. Check that Calibrators and samples have been correctly added. Then incubate the microplate at +37°C for 60 min.
3. Wash the microplate as reported in section I.3.
4. In all the wells except A1, pipette 100 µl Enzyme Conjugate. Check that the reagent has been correctly added. Incubate the microplate at +37°C for 60 minutes.

Important note:

- 3) Be careful not to touch the inner surface of the well with the pipette tip when dispensing the Enzyme Conjugate. Contamination might occur.
- 4) Mix thoroughly the Enzyme Conjugate on vortex before use.
5. Wash the microplate as described.
6. Pipette 100µl TMB/H₂O₂ mixture in each well, the blank wells included. Check that the reagent has been correctly added. Then incubate the microplate at room temperature for 20 minutes.

Important note: Do not expose to strong direct light as a high background might be generated.

7. Stop the enzymatic reaction by pipette 100µl Sulphuric Acid into each well and using the same pipetting sequence as in step 6. Then measure the colour intensity with a microplate reader at 450nm (reading) and at 620-630nm (blanking, strongly recommended), blanking the instrument on A1 and B1 wells.

Important general notes:

1. If the second filler is not available, ensure that no finger prints are present on the bottom of the microwell before reading at 450nm. Finger prints could generate false positive results on reading.
2. Reading has to be performed immediately after the addition of the Stop Solution but definitely no longer than 20 minutes afterwards. Some self oxidation of the chromogen can occur leading to a higher background.
3. The Control Serum (CS) does not affect the cut-off calculation and therefore the test results calculation. The Control Serum may be used only when a laboratory internal quality control is required by the management.

N. ASSAY SCHEME (standard procedure)

Specimen Diluent	50 µl
Calibrators	100 µl
Control Serum	100 µl
Samples	100 µl
1 st incubation	60 min
Temperature	+37°C
Wash step	4-5 cycles
Enzyme Conjugate	100 µl
2 nd incubation	60 min
Temperature	+37°C
Wash step	4-5 cycles
TMB/H ₂ O ₂ mix	100 µl
3 rd incubation	20 min
Temperature	rt
Sulphuric Acid	100 µl
Reading OD	450nm & 620nm

An example of dispensation scheme in quantitative assays is reported below:

Microplate											
1	2	3	4	5	6	7	8	9	10	11	12
A	BLK	CAL4	S3								
B	BLK	CAL4	S4								
C	CAL1	CAL5	S5								
D	CAL1	CAL5	S6								
E	CAL2	CS	S7								
F	CAL2	CS	S8								
G	CAL3	S1	S9								
H	CAL3	S2	S10								

Sample

Legenda: BLK = Blank // CAL = Calibrators // CS = Control Serum // S =

An example of dispensation scheme in qualitative assays is reported below:

Microplate											
1	2	3	4	5	6	7	8	9	10	11	12
A	BLK	S3	S11								
B	CAL1	S4	S13								
C	CAL1	S5	S13								
D	CAL2	S7	S15								
E	CAL2	S8	S16								
F	CAL3	S9	S17								
G	S1	S8	S17								
H	S2	S10	S18								

Legenda: BLK = Blank // CAL = Calibrators // S = Sample

O. INTERNAL QUALITY CONTROL

A validation check is carried out on the controls any time the kit is used in order to verify whether the performances of the assay are as qualified.

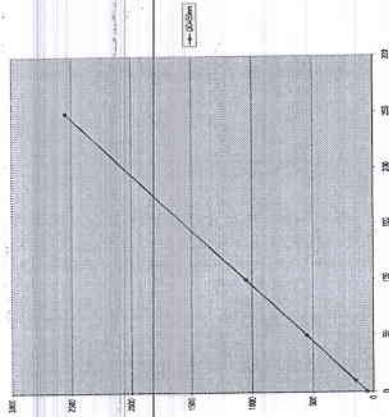
Control that the following data are matched:

Parameters	Requirements
Blank well	< 0.100 OD450nm
Calibrator 0 WHO mIU/ml	< 0.200 OD450nm after blanking
Calibrator 10 WHO mIU/ml	OD50nm higher than the OD450nm of the Calibrator 0 mIU/ml + 0.100
Calibrator 250 WHO mIU/ml	> 1.300 OD450nm
Control Serum	OD450nm = OD450nm CAL 30 mIU/ml ± 10%
Coefficient of variation	< 30% for the Calibrator 0 mIU/ml

If the results of the test match the requirements stated above, proceed to the next section, if they do not, do not proceed any further and perform the following checks:

Problem	Check
Blank well > 0.100 OD450nm	1. that the Chromogen/Substrate solution has not become contaminated during the assay
Calibrator 0 mIU/ml > 0.200	1. that the washing procedure and the washer settings are as validated in the pre qualification study.
Coefficient of variation > 30%	2. that the proper washing solution has been used and the washer has been primed with it before use.
	3. that no mistake has been done in the assay procedure when the dispensation of standards is carried out;
	4. that no contamination of the Cal 0 mIU/ml or of the wells where it was dispensed has occurred due to positive samples, to spills or to the enzyme conjugate;
	5. that micropipettes have not become contaminated with positive samples or with the enzyme conjugate
	6. that the washer needles are not blocked or partially obstructed.

Example of Calibration Curve :



Important Note:
Do not use the calibration curve above to make calculations.

P.2 Qualitative method

In the qualitative method, calculate the mean OD450nm values for the Calibrators 0 and 10 mIU/ml and then check that the assay is valid.

Example of calculation:

The following data must not be used instead of real figures obtained by the user.

Calibrator 0 mIU/ml: 0.020 - 0.024 OD450nm

Mean Value: 0.022 OD450nm

Lower than 0.200 - Accepted

Calibrator 10 mIU/ml: 0.250 - 0.270 OD450nm

Mean Value: 0.260 OD450nm

Higher than Cal 0 + 0.100 - Accepted

Calibrator 250 mIU/ml: 2.845 OD450nm

Higher than 1.500 - Accepted

Q. INTERPRETATION OF RESULTS

Samples with a concentration lower than 10 WHO mIU/ml are considered negative for anti HBsAg antibody by most of the international medical literature.

Samples with a concentration higher than 10 WHO mIU/ml are considered positive for anti HBsAg antibody.

In the follow up of vaccination recipients, however, the value of 20 WHO mIU/ml is usually accepted by the medical literature as the minimum concentration at which the patient is considered clinically protected against HBV infection.

Important notes:

1. Interpretation of results should be done under the supervision of the laboratory supervisor to reduce the risk of judgement errors and misinterpretations.
2. When test results are transmitted from the laboratory to another facility, attention must be paid to avoid erroneous data transfer.
3. Diagnosis has to be done and released to the patient by a suitably qualified medical doctor.

P. RESULTS

P.1 Quantitative method

If the test turns out to be valid, use for the quantitative method an approved curve fitting program to draw the calibration curve from the values obtained by reading at 450nm (4-parameters interpolation is suggested).

Then on the calibration curve calculate the concentration of anti HBsAg antibody in samples.

An example of Calibration curve is reported in the next page.

R. PERFORMANCES

Evaluation of Performances has been conducted in accordance to what reported in the Common Technical Specifications or CTS (art. 5, Chapter 3 of IVD Directive 98/79/EC).

1. LIMIT OF DETECTION

The limit of detection of the assay has been calculated by means of the HBsAg international preparation supplied by CLB on behalf of WHO (1st reference preparation 1977, lot 17-2-77), on which Calibration Curve has been calibrated. HBV negative Serum was used as diluent, as recommended by the supplier. Results of Quality Control are given in the following table:

WHO mIU/ml	SAB/CE Lot # 1002	SAB/CE Lot # 1001	SAB/CE Lot # 1002/22
50	0.933	0.812	0.846
10	0.219	0.192	0.194
5	0.110	0.096	0.104
2.5	0.057	0.058	0.067
Std 0	0.021	0.015	0.023

2. DIAGNOSTIC SPECIFICITY AND SENSITIVITY

A Performance Evaluation has been conducted on a total number of more than 700 samples.

2.1 Diagnostic Specificity

It is defined as the probability of the assay of scoring negative in the absence of specific analyte.

More than 500 negative specimens were tested, internally and externally, against a European company.

A diagnostic specificity of 96.8% was assessed.

Moreover, diagnostic specificity of other infectious diseases, potentially interfering specimens (other infectious diseases, patients affected by non viral hepatic diseases, dialysis patients, pregnant women, hemolized, icteric, etc.) against the European company. A value of specificity of 100% was assessed.

Finally, both human plasma, derived with different standard techniques of preparation (citrate, EDTA and heparin), and human sera have been used to determine the specificity.

No false reactivity due to the method of specimen preparation has been observed.

2.2 Diagnostic Sensitivity

It is defined as the probability of the assay of scoring positive in the presence of specific analyte.

106 vaccinated patients were evaluated providing a diagnostic sensitivity of 100%.

More than 100 HBV naturally infected patients were tested, internally and externally, against the European company, a diagnostic sensitivity of 100% was found.

3. PRECISION

The mean values obtained from a study conducted on three samples of different anti-HBsAg reactivity, examined in 16 replicates in three separate runs is reported below.

SAB/CE: lot # 1202

Calibrator 0 mIU/ml (N = 16)				
Mean values	1 st run	2 nd run	3 rd run	Average value
OD 450nm	0.038	0.038	0.039	0.039
Std Deviation	0.003	0.004	0.005	0.004
CV %	8.8	9.5	11.8	10.0

Calibrator 10 mIU/ml (N = 16)				
Mean values	1 st run	2 nd run	3 rd run	Average value
OD 450nm	0.250	0.243	0.244	0.246
Std Deviation	0.020	0.023	0.017	0.020
CV %	8.0	9.3	7.0	8.1

Calibrator 250 mIU/ml (N = 16)

Mean values	1 st run	2 nd run	3 rd run	Average value
OD 450nm	2.998	3.000	3.259	3.085
Std Deviation	0.152	0.151	0.158	0.153
CV %	5.1	5.0	4.8	5.0

SAB/CE: lot # 1002

Calibrator 0 mIU/ml (N = 16)

Mean values	1 st run	2 nd run	3 rd run	Average value
OD 450nm	0.048	0.048	0.050	0.049
Std Deviation	0.005	0.004	0.006	0.005
CV %	9.4	8.4	11.5	9.8

Calibrator 10 mIU/ml (N = 16)

Mean values	1 st run	2 nd run	3 rd run	Average value
OD 450nm	0.249	0.252	0.242	0.248
Std Deviation	0.021	0.020	0.023	0.021
CV %	8.3	7.9	9.6	8.6

Calibrator 250 mIU/ml (N = 16)

Mean values	1 st run	2 nd run	3 rd run	Average value
OD 450nm	3.544	3.653	3.612	3.603
Std Deviation	0.153	0.176	0.138	0.156
CV %	4.3	4.8	3.8	4.3

SAB/CE: lot # 1002/22

Calibrator 0 mIU/ml (N = 16)

Mean values	1 st run	2 nd run	3 rd run	Average value
OD 450nm	0.050	0.051	0.050	0.050
Std Deviation	0.005	0.006	0.006	0.005
CV %	10.0	10.9	11.9	10.9

Calibrator 10 mIU/ml (N = 16)

Mean values	1 st run	2 nd run	3 rd run	Average value
OD 450nm	0.226	0.238	0.239	0.234
Std Deviation	0.015	0.017	0.018	0.016
CV %	6.5	7.0	7.5	7.0

Calibrator 250 mIU/ml (N = 16)

Mean values	1 st run	2 nd run	3 rd run	Average value
OD 450nm	3.526	3.457	3.499	3.494
Std Deviation	0.137	0.143	0.162	0.147
CV %	3.9	4.1	4.6	4.2

The variability shown in the tables did not result in sample misclassification.

4. ACCURACY

The assay accuracy has been checked by the dilution and recovery tests. Any 'hook effect', underestimation likely to happen at high doses of analyte, was ruled out up to 10.000 mIU/ml.

S. LIMITATIONS OF THE PROCEDURE

Bacterial contamination or heat inactivation of the specimen may affect the absorbance values of the samples with consequent alteration of the level of the analyte. This test is suitable only for testing single samples and not pooled ones. Diagnosis of an infectious disease should not be established on the basis of a single test result. The patient's clinical history, symptomatology, as well as other diagnostic data should be considered.

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All the IVD Products manufactured by the company are under the control of a certified Quality Management System approved by an EC Notified Body. Each lot is submitted to a quality control and released into the market only if conforming with the EC technical specifications and acceptance criteria.

Manufacturer:

Dia Pro Diagnostic Bioprobes Srl
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HBcAb

**Competitive Enzyme Immunoassay for
the determination of antibodies
to Hepatitis B core Antigen
in human serum and plasma**

- for "in vitro" diagnostic use only -



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REF. BCAB.CE
96 Tests

HBcAb

A. INTENDED USE

Competitive Enzyme Immunoassay (ELISA) for the determination of antibodies to Hepatitis B core Antigen in human plasma and sera.

The kit is intended for the screening of blood units and the follow-up of HBV-infected patients.
For "in vitro" diagnostic use only.

B. INTRODUCTION

The World Health Organization (WHO) defines Hepatitis B as follows:

"Hepatitis B is one of the major diseases of mankind and is a serious global public health problem. Hepatitis means inflammation of the liver, and the most common cause is infection with one of 5 viruses, called hepatitis A, B, C, D, and E. All of these viruses can cause an acute disease with symptoms lasting several weeks including yellowing of the skin and eyes (jaundice); dark urine; extreme fatigue; nausea, vomiting and abdominal pain. It can take several months to a year to feel fit again. Hepatitis B virus can cause chronic infection in which the patient never gets rid of the virus and many years later develops cirrhosis of the liver or liver cancer."

HBV is the most serious type of viral hepatitis and the only type causing chronic hepatitis for which a vaccine is available. Hepatitis B virus is transmitted by contact with blood or body fluids of an infected person in the same way as human immunodeficiency virus (HIV), the virus that causes AIDS. However, HBV is 50 to 100 times more infectious than HIV. The main ways of getting infected with HBV are: (a) perinatal (from mother to baby at the birth); (b) child-to-child transmission; (c) unsafe injections and transfusions; (d) sexual contact.

Worldwide, most infections occur from infected mother to child, from child to child contact in household settings, and from reuse of unsterilized needles and syringes. In many developing countries, almost all children become infected with the virus. In many industrialized countries (e.g. Western Europe and North America), the pattern of transmission is different. In these countries, mother-to-infant and child-to-child transmission accounted for up to one third of chronic infections before childhood hepatitis B vaccination programmes were implemented. However, the majority of infections in these countries are acquired during young adulthood by sexual activity, and injecting drug use. In addition, hepatitis B virus is the major infectious occupational hazard of health workers, and most health care workers have received hepatitis B vaccine.

Hepatitis B virus is not spread by contaminated food or water, and cannot be spread casually in the workplace. High rates of chronic HBV infection are also found in the southern parts of Eastern and Central Europe. In the Middle East and Indian subcontinent, about 5% are chronically infected. Infection is less common in Western Europe and North America, where less than 1% are chronically infected.

Young children who become infected with HBV are the most likely to develop chronic infection. About 90% of infants infected during the first year of life and 30% to 50% of children infected between 1 to 4 years of age develop chronic

infection. The risk of death from HBV-related liver cancer or cirrhosis is approximately 25% for persons who become chronically infected during childhood.

Chronic hepatitis B in some patients is treated with drugs called *interferon* or *lamivudine*, which can help some patients. Patients with cirrhosis are sometimes given liver transplants, with varying success. It is preferable to prevent this disease with vaccine than to try and cure it.

Hepatitis B vaccine has an outstanding record of safety and effectiveness. Since 1982, over one billion doses of hepatitis B vaccine have been used worldwide. The vaccine is given as a series of three intramuscular doses. Studies have shown that the vaccine is 95% effective in preventing children and adults from developing chronic infection if they have not yet been infected. In many countries where 8% to 15% of children used to become chronically infected with HBV, the rate of chronic infection has been reduced to less than 1% in immunized groups of children. Since 1991, WHO has called for all countries to add hepatitis B vaccine into their national immunization programmes."

Hepatitis B core Antigen (or HBcAg) is the major component of the core particles of HBV.

HBcAg is composed of a single polypeptide of about 17 kD that is released upon disaggregating the core particles; the antigen contains at least one immunological determinant.

Upon primary infection, anti HBcAg antibodies are one of the first markers of HBV hepatitis appearing in the serum of the patient, slightly later than HBsAg, the viral surface antigen.

Anti HBcAg antibodies are produced usually at high titers and their presence is detectable even years after infection. Isolated HBcAb, in absence of other HBV markers, have been observed in infected blood units, suggesting the use of this test for screening HBV, in addition of HBsAg.

The determination of HBcAb has become important for the classification of the viral agent, together with the detection of the other markers of HBV infection, in sera and plasma.

C. PRINCIPLE OF THE TEST

The assay is based on the principle of competition where the antibodies in the sample compete with a monoclonal antibody for a fixed amount of antigen on the solid phase.

A purified recombinant HBcAg is coated to the microwell together with an additive able to block interferences present in the sample.

In the second incubation after washing, a monoclonal antibody, conjugated with Horseradish Peroxidase (HRP) and specific for HBcAg is added and binds to the free re-HBcAg coated on the plastic.

After incubation, microwells are washed to remove any unbound conjugate and then the chromogen/substrate is added. In the presence of peroxidase enzyme the colorless substrate is hydrolyzed to a colored end-product.

The color intensity is inversely proportional to the amount of antibodies to HBcAg present in the sample.

D. COMPONENTS

Each kit contains sufficient reagents to perform 96 tests.

1. Microplate MICROPLATE

8x12 microwell strips coated with recombinant HBcAg and sealed into a bag with desiccant. Allow the microplate to reach room temperature before opening; reseal unused strips in the bag with desiccant and store at 2-8°C.

F. WARNINGS AND PRECAUTIONS

- The kit has to be used by skilled and properly trained medical personnel only, under the supervision of a technical director responsible of the laboratory.
- When the kit is used for the screening of blood units and blood components, it has to be used in a laboratory certified and qualified by the national authority in that field (Ministry of Health or similar entity) to carry out this type of analysis.
- All the personnel involved in performing the assay have to wear protective laboratory clothes, lab-free gloves and glasses. The use of any sharp (needles) or cutting (blades) devices should be avoided. All the personnel involved should be trained in biosafety procedures, as recommended by the Center for Disease Control, Atlanta, U.S. and reported in the National Institute of Health's publication: "Biosafety in Microbiological and Biomedical Laboratories", ed. 1984.
- All the personnel involved in sample handling should be vaccinated for HBV and HAV, for which vaccines are available, safe and effective.
- The laboratory environment should be controlled so as to avoid contaminants such as dust or air-borne microbial agents, when opening kit vials and microplates and when performing the test. Protect the Chromogen (TMB) from strong light and avoid vibration of the bench surface where the test is undertaken.
- Upon receipt, store the kit at 2-8°C into a temperature controlled refrigerator or cold room.
- Do not interchange components between different lots of the kits. It is recommended that components between two kits of the same lot should not be interchanged.
- Check that the reagents are clear and do not contain visible large particles or aggregates. If not, advise the laboratory supervisor to initiate the necessary procedures.
- Avoid cross-contamination between serum/plasma samples by using disposable tips and changing them after each sample.
- Avoid cross-contamination between kit reagents by using disposable tips and changing them between the use of each one.
- Do not use the kit after the expiration date stated on external (primary container) and internal (vials) labels.
- Treat all specimens as potentially infective. All human serum specimens should be handled at Biosafety Level 2, as recommended by the Center for Disease Control, Atlanta, U.S. in compliance with what reported in the Institutes of Health's publication: "Biosafety in Microbiological and Biomedical Laboratories", ed. 1984.
- The use of disposable plastic-ware is recommended in the preparation of the washing solution or in transferring components into other containers of automated workstations, in order to avoid contamination.
- Waste produced during the use of the kit has to be discarded in compliance with national directives and laws concerning laboratory waste of chemical and biological substances. In particular, liquid waste generated from the washing procedure, from residuals of controls and from samples has to be treated as potentially infective material and inactivated. Suggested procedures of inactivation are treatment with a 10% final concentration of household bleach for 16-18 hrs or heat inactivation by autoclave at 121°C for 20 min.
- Accidental spills have to be adsorbed with paper tissues soaked with household bleach and then with water. Tissues should then be discarded in proper containers designated for laboratory/hospital waste.
- The Sulphuric Acid is an irritant. In case of spills, wash the surface with plenty of water.
- Other waste materials generated from the use of the kit (example: tips used for samples and controls, used microplates) should be handled as potentially infective and discarded.

2. Negative Control **CONTROL -**

1x1.0ml/vial. Ready to use. Contains 5% bovine serum albumin, 10 mM phosphate buffer pH 7.4 +/-0.1, 0.09% sodium azide and 0.1% Kathon GC as preservatives. The negative control is pale yellow color coded.

3. Positive Control **CONTROL +**

1x1.0ml/vial. Ready to use. Contains 5% bovine serum albumin, anti HbC α g antibodies at a concentration of about 10 PEI U/ml, calibrated on PEI HbC Reference Material 82), 10 mM phosphate buffer pH 7.4 +/-0.1, 0.09% sodium azide and 0.1% Kathon GC as preservatives. The positive control is green color coded.

4. Calibrator **CAL ...**

n°1 vial. Lyophilized. To be dissolved with EIA grade water as reported in the label. Contains fetal bovine serum, human antibodies to HbC α g at a concentration of 2 PEI U/ml +/-10% (calibrated on PEI HbC Reference Material 82) and 0.1% Kathon GC as preservative.

Note: The volume necessary to dissolve the content of the vial may vary from lot to lot. Please use the right volume reported on the label.

5. Wash buffer concentrate **WASHBUF 20X**

1x60ml/bottle. 20x concentrated solution. Once diluted, the wash solution contains 10 mM phosphate buffer pH 7.0 +/-0.2, 0.05% Tween 20 and 0.1% Kathon GC.

6. Enzyme Conjugate **CONJ**

1x15ml/vial. Ready-to-use solution. Contains 5% bovine serum albumine, 10 mM tris buffer pH 6.8 +/-0.1, Horseradish peroxidase conjugated mouse monoclonal antibody to HbC α g in presence of 0.3 mg/ml gentamicin sulphate and 0.1% Kathon GC as preservatives. The component is red colour coded.

7. Chromogen/Substrate **SUBS TMB**

1x15ml/vial. Contains a 50 mM citrate-phosphate buffered solution at pH 3.6 +/-0.1, 0.03% tetra-methyl-benzidine (TMB), 0.02% hydrogen peroxide (H₂O₂) and 4% dimethylsulphoxide **Note:** To be stored protected from light as sensitive to strong illumination.

8. Specimen Diluent **DILSPD**

4x3ml/vial. 10 mM tris buffered solution pH 8.0 +/-0.1 containing 0.1% Kathon GC for the pre-treatment of samples and controls in the plate, blocking interference.

Note: Use all the content of one vial before opening a second one. The reagent is sensitive to oxidation.

9. Sulphuric Acid **H₂SO₄ 0.3M**

1x15ml/vial. Contains 0.3 M H₂SO₄ solution. Attention: Irritant (Xi R36/38; S2/26/30)

10. Plate sealing foil n°2

11. Instruction manual n°1

F. MATERIALS REQUIRED BUT NOT PROVIDED

- Calibrated Micropipettes (100ul and 50ul) and disposable plastic tips.
- EIA grade water (double distilled or deionised, charcoal treated to remove oxidizing chemicals used as disinfectants).
- Timer with 60 minute range or higher.
- Absorbent paper tissues.
- Calibrated ELISA microplate thermostatic incubator (dry or wet) set at +37°C.
- Calibrated ELISA microwell reader with 450nm (reading) and with 620-630nm (blanking) filters.
- Calibrated ELISA microplate washer.
- Vortex or similar mixing tools.

discarded according to national directives and laws concerning laboratory wastes.

G. SPECIMEN: PREPARATION AND RECOMMENDATIONS

- Blood is drawn aseptically by venepuncture and plasma or serum is prepared using standard techniques of preparation of samples for clinical laboratory analysis. No influence has been observed in the preparation of the sample with citrate, EDTA and heparin.
- Avoid any addition of preservatives to samples; especially sodium azide as this chemical would affect the enzymatic activity of the conjugate.
- Samples have to be clearly identified with codes or names in order to avoid misinterpretation of results. When the kit is used for the screening of blood units, bar code labeling and electronic reading is strongly recommended.
- Haemolysed (red) and visibly hyperlipemic ("milky") samples have to be discarded as they could generate false results. Samples containing residues of fibrin or heavy particles or microbial filaments and bodies should be discarded as they could give rise to false results.
- Sera and plasma can be stored at +2°-8°C for up to five days after collection. For longer storage periods, samples can be stored frozen at -20°C for several months. Any frozen samples should not be frozen/thawed more than once as this may generate particles that could affect the test result.
- If particles are present, centrifuge at 2 000 rpm for 20 min or filter using 0.2-0.8u filters to clean up the sample for testing.

H. PREPARATION OF COMPONENTS AND WARNINGS

A study conducted on an opened kit has not pointed out any relevant loss of activity up to 6 re-uses of the device and up to 6 months.

1. Microplates:

Allow the microplate to reach room temperature (about 1 hr) before opening the container. Check that the desiccant has not turned dark green, indicating a defect in storage.

In this case, call Dia.Pro's customer service. Unused strips have to be placed back inside the aluminum pouch, with the desiccant supplied, firmly zipped and stored at +2°-8°C. After first opening, remaining strips are stable until the humidity indicator inside the desiccant bag turns from yellow to green.

2. Negative Control:

Ready to use. Mix well on vortex before use.

3. Positive Control:

Ready to use. Mix well on vortex before use.

4. Calibrator:

Add the volume of ELISA grade water, reported on the label, to the lyophilised powder; let fully dissolve and then gently mix on vortex.

Note: The dissolved calibrator is not stable. Store it frozen in aliquots at -20°C.

5. Wash buffer concentrate:

The whole content of the concentrated solution has to be diluted 20x with bidistilled water and mixed gently end-over-end before use. During preparation avoid foaming as the presence of bubbles could impact on the efficiency of the washing cycles.

Note: Once diluted, the wash solution is stable for 1 week at +2°-8°C.

6. Enzyme conjugate:

Ready to use. Mix well on vortex before use. Avoid contamination of the liquid with oxidizing chemicals, dust or microbes. If this component has to be transferred, use only plastic, and if possible, sterile disposable containers.

7. Chromogen/Substrate:

Ready to use. Mix well on vortex before use. Avoid contamination of the liquid with oxidizing chemicals, air-borne dust or microbes. Do not expose to strong light, oxidizing agents and metallic surfaces. If this component has to be transferred use only plastic, and if possible, sterile disposable container.

8. Specimen Diluent:

Ready to use solution. Mix gently on vortex before use. Use all the content of one vial before opening a second one. The reagent is sensitive to oxidation.

9. Sulphuric Acid:

Ready to use. Mix well on vortex before use. Attention: Irritant (Xi R36/38; S2/26/30) Legend: R 36/38 = Irritating to eyes and skin, S 2/26/30 = In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.

I. INSTRUMENTS AND TOOLS USED IN COMBINATION

WITH THE KIT

- Micropipettes have to be calibrated to deliver the correct volume required by the assay and must be submitted to regular decontamination (70% ethanol, 10% solution of bleach, hospital grade disinfectants) of those parts that could accidentally come in contact with the sample or the components of the kit. They should also be regularly maintained in order to show a precision of 1% and a linearity of +2%.
- The ELISA incubator has to be set at +37°C (tolerance of +0.5°C) and regularly checked to ensure the correct temperature is maintained. Both dry incubators and water baths are suitable for the incubations, provided that the instrument is validated for the incubation of ELISA tests.
- The ELISA washer is extremely important to the overall performance of the assay. The washer must be carefully validated and correctly optimized using the kit controls/calibrator and reference panels, before using the kit for routine laboratory tests. Usually 4-5 washing cycles (aspiration + dispensation of 350 ul/well of washing solution = 1 cycle) are sufficient to ensure that the assay performs as expected. A soaking time of 20-30 seconds between cycles is suggested. In order to set correctly their number, it is recommended to run an assay with the kit controls/calibrator and well characterized negative and positive reference samples, and check to match the values reported below in the sections "Validation of Test" and "Assay Performances". Regular calibration of the volumes delivered and maintenance (decontamination and cleaning of needles) of the washer has to be carried out according to the instructions of the manufacturer.
- The ELISA microplate reader has to be equipped with a reading filter of 450nm and with a second filter 620-630nm, strongly recommended for blanking purposes. Its standard performances should be (a) bandwidth ≤ 10 nm; (b) absorbance range from 0 to ≥ 2.0 ; (c) linearity $\Delta A \geq 2.0 \times 10^{-3}$; repeatability $\geq 1\%$. Blanking is carried out on the well identified in the section "Assay Procedure". The optical system of the reader has to be calibrated regularly to ensure that the correct optical density is measured. It should be regularly maintained according to the manufacturer's instructions.

6. When using an ELISA automated work station, all critical steps (dispensation, incubation, washing, reading, shaking, data handling) have to be carefully set, calibrated, controlled and regularly serviced in order to match the values reported in the sections "Validation of Test" and "Assay Performances". The assay protocol has to be installed in the operating system of the unit and validated as for the washer and the reader. In addition, the liquid handling part of the station (dispensation and washing) has to be validated and correctly set. Particular attention must be paid to avoid carry over by the needles used for dispensing samples and for washing. This must be studied and controlled to minimize the possibility of contamination of adjacent wells due to strongly reactive samples, leading to false positive results. The use of ELISA automated work stations is recommended for blood screening and when the number of samples to be tested exceed 20-30 units per run.
7. Dia.Pro's customer service offers support to the user in the setting and checking of instruments used in combination with the kit, in order to assure full compliance with the requirements described. Support is also provided for the installation of new instruments to be used with the kit.

L. PRE ASSAY CONTROLS AND OPERATIONS

1. Check the expiration date of the kit printed on the external label (primary container). Do not use if expired.
2. Check that the liquid components are not contaminated by visible particles or aggregates. Check that the Chromogen (TMB) is colourless or pale blue by aspirating a small volume of it with a sterile plastic pipette. Check that no leakage occurred in transportation and no spillage of liquid is present inside the box (primary container). Check that the aluminium pouch, containing the microplate, is not punctured or damaged.
3. Dilute all the content of the 20x concentrated Wash Solution as described above.
4. Dissolve the Calibrator as described above and gently mix.
5. Allow all the other components to reach room temperature (about 1 hr) and then mix gently on vortex all liquid reagents.
6. Set the ELISA incubator at +37°C and prepare the ELISA washer by priming with the diluted washing solution, according to the manufacturers instructions. Set the right number of washing cycles as found in the validation of the instrument for its use with the kit.
7. Check that the ELISA reader is turned on or ensure it will be turned on at least 20 minutes before reading.
8. If using an automated work station, turn on, check settings and be sure to use the right assay protocol.
9. Check that the micropipettes are set to the required volume.
10. Check that all the other equipment is available and ready to use.
11. In case of problems, do not proceed further with the test and advise the supervisor.

M. ASSAY PROCEDURE

The assay has to be performed according to the procedure given below, taking care to maintain the same incubation time for all the samples being tested.

1. Place the required number of strips in the plastic holder and carefully identify the wells for controls, calibrator and samples.
2. Leave the A1 well empty for blanking purposes.
3. Dispense 50 µl Specimen Diluent into all the control and sample wells.
4. Pipette 50 µl of the Negative Control in triplicate, 50 µl of the Calibrator in duplicate and then 50 µl of the Positive Control in single. Then dispense 50 µl of each of the samples.
5. Incubate the microplate for 60 min at +37°C.

Important note: Strips have to be sealed with the adhesive sealing foil, only when the test is performed manually. Do not cover strips when using ELISA automatic instruments.

6. When the first incubation is finished, wash the microwells as previously described (section I.3).
7. Pipette 100 µl Enzyme Conjugate in all the wells, except A1; incubate the microplate for 60 min at +37°C.

Important note: Be careful not to touch the plastic inner surface of the well with the tip filled with the Enzyme Conjugate. Contamination might occur.

8. When the second incubation is finished, wash the microwells as previously described (section I.3).
9. Pipette 100 µl Chromogen/Substrate into all the wells, A1 included.

Important note: Do not expose to strong direct light, as a high background might be generated.

10. Incubate the microplate protected from light at room temperature (18-24°C) for 20 minutes. Wells dispensed with negative control and negative samples will turn from clear to blue (competitive method).
11. Pipette 100 µl Sulphuric Acid into all the wells using the same pipetting sequence as in step 9 to stop the enzymatic reaction. Addition of the stop solution will turn the negative control and negative samples from blue to yellow.
12. Measure the colour intensity of the solution in each well, as described in section I.5 using a 450nm filter (reading) and a 620-630nm filter (background subtraction, strongly recommended), blanking the instrument on A1.

Important notes:

1. If the second filter is not available, ensure that no finger prints are present on the bottom of the microwell before reading at 450nm. Finger prints could generate false positive results on reading.
2. Reading has to be performed immediately after the addition of the Stop Solution but definitely no longer than 20 minutes afterwards. Some self oxidation of the chromogen can occur leading to a higher background.

N. ASSAY SCHEME

Specimen Diluent	50 µl
Controls/calibrator and samples	50 µl
1 st incubation	60 min
Temperature	+37°C
Wash	n°4-5
Enzyme Conjugate	100 µl
2 nd incubation	60 min
Temperature	+37°C
Wash	n°4-5
TMB-H2O2 mix	100 µl
3 rd incubation	20 min
Temperature	r.t.
Sulphuric Acid	100 µl
Reading OD	450nm

An example of dispensation scheme is reported below:

Microplate

	1	2	3	4	5	6	7	8	9	10	11	12
A	BLK	S2										
B	NC	S3										
C	NC	S4										
D	NC	S5										
E	CAL	S6										
F	CAL	S7										
G	PC	S8										
H	ST	S9										

Legend: BLK = Blank NC = Negative Control S = Sample
CAL = Calibrator PC = Positive Control

O. INTERNAL QUALITY CONTROL

A check is performed on the controls/calibrator any time the kit is used in order to verify whether the expected OD450nm or Co/S values have been matched in the analysis. Ensure that the following parameters are met:

Parameter	Requirements
Blank well	< 0.050 OD450nm value
Negative Control (NC)	> 1.000 OD450nm after blanking coefficient of variation < 20%
Calibrator (about 2 PEI U/ml)	Co/S > 1
Positive Control	< 0.200 OD450nm

If the results of the test match the requirements stated above, proceed to the next section.

If they do not, do not proceed any further and perform the following checks:

Problem	Check
Blank well > 0.050 OD450nm	that the Chromogen/Substrate solution has not become contaminated during the assay
Negative Control (NC) < 1.000 OD450nm after blanking coefficient of variation > 20%	1. that the washing procedure and the washer settings are as validated in the pre qualification study; 2. that the proper washing solution has been used and the washer has been primed with this solution; 3. that the washing procedure and the washer settings are as validated in the pre qualification study; 4. that no external contamination of the calibrator has occurred; 5. that microplate has been correctly performed; 6. that no mistake has occurred during the distribution of the control (dispensation of negative control instead of the control); 7. that the washing procedure and the washer settings are as validated in the pre qualification study; 8. that no external contamination of the positive control has occurred.
Calibrator Co/S < 1	1. that the procedure has been correctly performed; 2. that no mistake has occurred during the distribution of the control (dispensation of negative control instead of the control); 3. that the washing procedure and the washer settings are as validated in the pre qualification study; 4. that no external contamination of the positive control has occurred.
Positive Control > 0.200 OD450nm	1. that the procedure has been correctly performed; 2. that no mistake has occurred during the distribution of the control (dispensation of negative control instead of the control); 3. that the washing procedure and the washer settings are as validated in the pre qualification study; 4. that no external contamination of the positive control has occurred.

If any of the above problems have occurred, report the problem to the supervisor for further actions.

P. RESULTS

The results are calculated by means of a cut-off value determined with the following formula:

Cut-Off = (NC + PC) / 5

Important note: When the calculation of results is performed by the operating system of an ELISA automated work station, ensure that the proper formula is used to calculate the cut-off value and generate the correct interpretation of results.

Q. INTERPRETATION OF RESULTS

Results are interpreted as ratio between the cut-off value and the sample OD450nm or Co/S.

Results are interpreted according to the following table:

Co/S	Interpretation
< 0.9	Negative
0.9 - 1.1	Equivocal
> 1.1	Positive

A negative result indicates that the patient has not been infected by HBV.
Any patient showing an equivocal result should be re-tested on a second sample taken 1-2 weeks after the initial sample.
The blood unit should not be transfused.
A positive result is indicative of HBV infection and therefore the patient should be treated accordingly or the blood unit should be discarded.

Important notes:

1. Interpretation of results should be done under the supervision of the laboratory supervisor to reduce the risk of judgement errors and misinterpretations.
2. When test results are transmitted from the laboratory to another facility, attention must be paid to avoid erroneous data transfer.
3. Diagnosis of viral hepatitis infection has to be taken by and released to the patient by a suitably qualified medical doctor.

An example of calculation is reported below.

The following data must not be used instead of real figures obtained by the user.

Negative Control: 2.000 - 2.200 - 2.000 OD450nm
Mean Value: 2.100 OD450nm
Higher than 1.000 - Accepted

Positive Control: 0.100 OD450nm
Lower than 0.200 - Accepted

Cut-Off = (2.100 + 0.100) / 5 = 0.440

Calibrator: 0.400-0.380 OD450nm
Mean Value: 0.380 OD450nm
Co/S > 1 - Accepted

Sample 1: 0.028 OD450nm
Sample 2: 1.890 OD450nm
Sample 1 Co/S > 1 positive
Sample 2 Co/S < 0.9 negative

R. PERFORMANCE

Evaluation of Performance has been conducted in accordance to what reported in the Common Technical Specifications or CTS (art. 5, Chapter 3 of IVD Directive 98/79/EC).

1. LIMIT OF DETECTION:

The sensitivity of the assay has been calculated by means of the reference preparation for HbCAB supplied by Paul Ehrlich Institute (PEI HbC Reference Material 82). The assay shows a sensitivity of about 1.25 PEI U/ml.
The table below reports the CoS values shown by the PEI standard diluted as suggested by the manufacturer to prepare a limiting dilution curve in Fetal Calf Serum (FCS).

PEI U/ml	Lot 1001	Lot 0702	Lot 07022	Lot 1202
5	22.5	18.0	19.0	17.7
2.5	8.0	5.5	5.4	5.0
1.25	1.1	1.3	1.0	1.0
0.625	0.4	0.4	0.4	0.4

In addition Accurin 1 – series 3000 – supplied by Boston Biomedica Inc., USA, was tested to determine its CoS value. Results are reported in the table below:

Accurin 1 – series 3000

Value	Lot 1001	Lot 0702	Lot 1202
CoS	2.9	2.3	2.2

2. DIAGNOSTIC SPECIFICITY AND SENSITIVITY

The Performance Evaluation of the device was carried out in a trial conducted on more than total 6000 samples.

2.1 Diagnostic Specificity

It is defined as the probability of the assay of scoring negative in the absence of specific analyte. A total of more 5000 unselected donors, including 1st time donors, were examined.
In a first study 2023 samples were tested against a US company as reference. A specificity of 98.5% was found. In a second study 1588 samples were examined against a European company. A specificity of 99.7% was found. In the last study 1565 samples were assayed against the same US company, a value of 99.8% was found.

In addition to the above population, 206 samples from hospitalized patients were tested against the European company. A value of 99.3% specificity was found.

Moreover, diagnostic specificity was assessed by testing 164 potentially interfering specimens (other infectious diseases, patients affected by non viral hepatic diseases, dialysis patients, pregnant women, hemolized, lipemic, etc.) against the European company. A value of specificity of 100% was assessed.
Finally, both human plasma, derived with different standard techniques of preparation (citrate, EDTA and heparin), and human sera have been used to determine the specificity.
No false reactivity due to the method of specimen preparation has been observed.

2.2 Diagnostic Sensitivity

It is defined as the probability of the assay of scoring positive in the presence of specific analyte.
373 positive specimens were tested against the European company, a diagnostic sensitivity of 99.7 was found.

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All the IVD Products manufactured by the company are under the control of a certified Quality Management System approved by an EC Notified Body. Each lot is submitted to a quality control and released into the market only if conforming with the EC technical specifications and acceptance criteria.

Produced by
Dia Pro Diagnostic Bioprobes Srl
Via G. Carducci n° 27 – Sesto San Giovanni (MI) – Italy

CE
0318

3. PRECISION

The mean values obtained from a study conducted on three lots and on two samples of different anti-HbCag reactivity, examined in 16 replicates in three separate runs is reported below.

BCAB CE: lot # 1202

Negative Control (N = 16)				
Mean values	1st run	2nd run	3 rd run	Average value
OD 450nm	1.943	1.939	1.924	1.935
Std.Deviation	0.081	0.076	0.103	0.087
CV %	4.2	4.0	5.3	4.5

Calibrator (N = 16)

Mean values	1st run	2nd run	3 rd run	Average value
OD 450nm	0.143	0.147	0.148	0.146
Std.Deviation	0.014	0.017	0.018	0.016
CV %	9.8	11.4	12.1	11.1
CoS	2.8	2.7	2.6	2.7

BCAB CE: lot # 0702

Negative Control (N = 16)				
Mean values	1st run	2nd run	3 rd run	Average value
OD 450nm	2.163	2.110	2.106	2.126
Std.Deviation	0.105	0.098	0.139	0.11
CV %	4.9	4.2	6.6	5.2

Calibrator (N = 16)

Mean values	1st run	2nd run	3 rd run	Average value
OD 450nm	0.162	0.193	0.195	0.190
Std.Deviation	0.018	0.023	0.019	0.020
CV %	10.0	12.0	9.9	10.6
CoS	2.5	2.2	2.3	2.3

BCAB CE: lot # 07022

Negative Control (N = 16)				
Mean values	1st run	2nd run	3 rd run	Average value
OD 450nm	2.278	2.098	2.130	2.169
Std.Deviation	0.135	0.126	0.159	0.140
CV %	5.9	6.0	7.5	6.5

Calibrator (N = 16)				
Mean values	1st run	2nd run	3 rd run	Average value
OD 450nm	0.193	0.190	0.199	0.194
Std.Deviation	0.023	0.023	0.027	0.025
CV %	12.1	12.3	13.5	12.6
CoS	2.4	2.2	2.2	2.3

The variability shown in the tables did not result in sample misclassification.

S. LIMITATIONS OF THE PROCEDURE

Bacterial contamination or heat inactivation of the specimen may affect the absorbance values of the samples with consequent alteration of the level of the analyte. This test is suitable only for testing single samples and not pooled ones.
Diagnosis of an infectious disease should not be established on the basis of a single test result. The patient's clinical history, symptomatology, as well as other diagnostic data should be considered.

HBe Ag&Ab

Enzyme Immunoassay (ELISA) for the determination of Hepatitis B Virus "e" Antigen and Antibody in human plasma and sera.

- for "in vitro" diagnostic use only -



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REF. HBE/CE
96 Tests

HBe Ag&Ab

A. INTENDED USE

Enzyme ImmunoAssay (ELISA) for the determination of Hepatitis B Virus "e" Antigen and Antibody in human plasma and sera.
The kit is intended for the follow-up of acute infection and of chronic patients under therapy.
For "in vitro" diagnostic use only.

B. INTRODUCTION

Hepatitis B "e" Antigen or HBeAg is known to be intimately associated with Hepatitis B Virus or HBV replication and the presence of infectious Dane particles in the blood.
Recently, it has been found that HBeAg is a product of proteolytic degradation of Hepatitis B core Antigen or HBeAg, occurring in hepatocytes, whose expression is under the control of the precore region of HBV genome.
If HBeAg is considered a specific marker of infectivity, the presence of anti HBeAg antibodies in blood is recognised to be a clinical sign of recovery from infection to convalescence.
The determination of these two analytes in samples from HBV patients has become important for the classification of the phase of illness and as a prognostic value in the follow up of infected patients.

C. PRINCIPLE OF THE TEST

HBeAg:
HBeAg, if present in the sample, is captured by a specific monoclonal antibody, in the 1st incubation.
In the 2nd incubation, after washing, a tracer, composed of a mix of two specific anti HBeAg monoclonal antibodies, labeled with peroxidase (HRP), is added to the microplate and binds to the captured HBeAg.
The concentration of the bound enzyme on the solid phase is proportional to the amount of HBeAg in the sample and its activity is detected by adding the chromogen/substrate in the 3rd incubation.
The presence of HBeAg in the sample is determined by means of a cut-off value that allows for the semiquantitative detection of the antigen.

HBeAb

Anti HBeAg antibodies, if present in the sample, compete with a recombinant HBeAg preparation for a fixed amount of an anti HBeAg antibody, coated on the microplate wells.

The competitive assay is carried out in two incubations, the first with the sample and reHBeAg, and the second with a tracer, composed of two anti HBeAg monoclonal antibodies, labeled with peroxidase (HRP).

The concentration of the bound enzyme on the solid phase becomes inversely proportional to the amount of anti HBeAg antibodies in the sample and its activity is detected by adding the chromogen/substrate in the third incubation.

The concentration of HBeAg specific antibodies in the sample is determined by means of a cut-off value that allows for the semi quantitative detection of anti HBeAg antibodies.

D. COMPONENTS

The kit contains reagents for total 96 tests.

1. Microplate/MICROPLATE

n° 1 coated microplate
12 strips of 8 breakable wells coated with anti HBeAg specific monoclonal antibody, postcoated with bovine serum proteins and sealed into a bag with desiccant. Allow the microplate to reach room temperature before opening, reseal unused strips in the bag with desiccant and store at 2-8 °C.

2. **Negative Control:** [CONTROL -]
1x2.0ml/vial. Ready to use control. It contains bovine serum, 0.09% sodium azide and 0.045% ProClin 300 as preservatives.
The negative control is colorless.

3. **Antigen Positive Control:** [CONTROL + Ag]
1x1.0ml/vial. Ready to use control. It contains 2% bovine serum albumin, non infectious recombinant HBeAg, 100 mM Tris buffer pH 7.4/-0.1, 0.09% sodium azide and 0.045% ProClin 300 as preservatives.
The positive control is green color coded.

4. **Antibody Positive Control:** [CONTROL + Ab]

1x1.0ml/vial. Ready to use control. It contains 2% bovine serum albumin, human anti HBeAg positive plasma at about 10 PEI U/ml, 100 mM Tris buffer pH 7.4/-0.1, 0.09% sodium azide and 0.045% ProClin 300 as preservatives. The label is red colored.
The positive control is yellow color coded.

5. **Antigen Calibrator:** [CALAB -m]

n° 1 vial. Lyophilized calibrator for HBeAg. To be dissolved with EIA grade water as reported in the label. It contains total bovine serum, non infectious recombinant HBeAg at 1 PEI U/ml +/- 10%, 0.02% gentamicine sulphate and 0.045% ProClin 300 as preservatives.

Important Note: The volume necessary to dissolve the content of the vial may vary from lot to lot. Please use the right volume reported on the label.

6. **Antibody Calibrator:** [CALAB -m]

n° 1 vial. Lyophilized calibrator for anti HBeAg antibody. To be dissolved with EIA grade water as reported in the label. It contains fetal bovine serum, positive plasma at 0.25 PEI U/ml +/- 10%, 0.02% gentamicine sulphate and 0.045% ProClin 300 as preservatives. The label is red colored.

Important Note: The volume necessary to dissolve the content of the vial may vary from lot to lot. Please use the right volume reported on the label.

7. **Wash buffer concentrate:** [WASHBUF 20X]

1x60ml/bottle. 20x concentrated solution.
Once diluted, the wash solution contains 10 mM phosphate buffer pH 7.0/-0.2, 0.05% Tween 20 and 0.045% ProClin 300.

8. **Enzyme conjugate:** [CONJ]

1x16ml/vial. Ready to use conjugate. It contains Horseradish peroxidase conjugated with a mix of monoclonal antibodies to HBeAg. 10 mM Tris buffer pH 6.8/-0.1, 2% BSA, 0.045% ProClin 300 and 0.02% gentamicine sulphate as preservatives.
The reagent is red color coded.

9. **HBe Antigen:** [Ag-HBe]

1x10ml/vial. Ready to use reagent. It contains recombinant HBeAg, fetal bovine serum, buffered solution pH 8.0/-0.1, 0.045% ProClin 300 and 0.09% sodium azide as preservatives.
The reagent is blue color coded.

10. **Chromogen/Substrate:** [SUBS TMB]

1x16ml/vial. Ready-to-use component. It contains a 50 mM citrate-phosphate buffered solution at pH 3.5-3.6, 4% dimethylsulphoxide, 0.03% tetra-methyl-benzidine or TMB and 0.02% hydrogen peroxide or H₂O₂.
Note: To be stored protected from light as sensitive to strong illumination.

11. **Sulphuric Acid:** [H₂SO₄ 0.3 M]

1x15ml/vial. It contains 0.3 M H₂SO₄ solution.
Attention: Irritant (H315, H319; P280, P302+P352, P332+P313, P305+P351+P338, P337+P313, P362+P363).

12. **Plate sealing foils** n°2

13. Package insert n°1

E. MATERIALS REQUIRED BUT NOT PROVIDED

1. Calibrated Micropipettes (150ul, 100ul and 50ul) and disposable plastic tips.
2. EIA grade water (double distilled or deionized, charcoal treated to remove oxidizing chemicals used as disinfectants).
3. Timer with 60 minute range or higher.
4. Absorbent paper tissues.
5. Calibrated ELISA microplate thermolabile incubator (dry or wet) set at +37°C.
6. Calibrated ELISA microwell reader with 450nm (reading) and with 620-630nm (blanking) filters.
7. Calibrated ELISA micropipette washer.
8. Vortex or similar mixing tools.

F. WARNINGS AND PRECAUTIONS

1. The kit has to be used by skilled and properly trained technical personnel only, under the supervision of a medical doctor responsible of the laboratory.
2. All the personnel involved in performing the assay have to wear protective laboratory clothes, face-free gloves and glasses. The use of any sharp (needles) or cutting (blades) devices should be avoided. All the personnel involved should be trained in biosafety procedures, as recommended by the Center for Disease Control, Atlanta, U.S. and reported in the National Institute of Health's publication: "Biosafety in Microbiological and Biomedical Laboratories", ed. 1984.
3. All the personnel involved in sample handling should be vaccinated for HBV and HAV, for which vaccines are available, safe and effective.
4. The laboratory environment should be controlled so as to avoid contaminants such as dust or air-borne microbial agents, when opening kit vials and microplates and when performing the test. Protect the Chromogen/Substrate (TMB) from strong light and avoid vibration of the bench surface where the test is undertaken.
5. Upon receipt, store the kit at 2-8°C into a temperature controlled refrigerator or cold room.
6. Do not interchange components between different lots of the kits. It is recommended that components between two lots of the same lot should not be interchanged.
7. Check that the reagents are clear and do not contain visible heavy particles or aggregates. If not, advise the laboratory supervisor to initiate the necessary procedures.
8. Avoid cross-contamination between serum/plasma samples by using disposable tips and changing them after each sample. Do not reuse disposable tips.
9. Avoid cross-contamination between kit reagents by using disposable tips and changing them between the use of each one. Do not reuse disposable tips.
10. Do not use the kit after the expiration date stated on external (primary container) and internal (vials) labels.
11. Treat all specimens as potentially infectious. All human serum specimens should be handled at Biosafety Level 2, as recommended by the Center for Disease Control, Atlanta, U.S. in compliance with what reported in the Institutes of Health's publication: "Biosafety in Microbiological and Biomedical Laboratories", ed. 1984.
12. The use of disposable plastic-ware is recommended in the preparation of the washing solution or in transferring components into other containers of automated workstations, in order to avoid contamination.
13. Waste produced during the use of the kit has to be discarded in compliance with national directives and laws concerning laboratory waste of chemical and biological substances. In particular, liquid waste generated from the washing procedure, from residuals of controls and from samples has to be treated as potentially infectious material and inactivated. Suggested procedures of inactivation are:

treatment with a 10% final concentration of household bleach for 16-18 hrs or heat inactivation by autoclave at 121°C for 20 min.

14. Accidental spills have to be absorbed with paper tissues soaked with household bleach and then with water. Tissues should then be discarded in proper containers designated for laboratory/hospital waste.
15. The Stop Solution is an irritant. In case of spills, wash the surface with plenty of water.
16. Other waste materials generated from the use of the kit (example: tips used for samples and controls, used microplates) should be handled as potentially infectious and disposed according to national directives and laws concerning laboratory wastes.

G. SPECIMEN: PREPARATION AND RECOMMENDATIONS

1. Blood is drawn aseptically by venepuncture and plasma or serum is prepared using standard techniques of preparation of samples for clinical laboratory analysis. No influence has been observed in the preparation of the sample with citrate, EDTA and heparin.
2. Avoid any addition of preservatives; especially sodium azide as this chemical would affect the enzymatic activity of the conjugate, generating false negative results.
3. Samples have to be clearly identified with codes or names in order to avoid misinterpretation of results.
4. Haemolysed and visibly hyperlipemic ("milky") samples have to be discarded as they could generate false results. Samples containing residues of fibrin or heavy particles or microbial filaments and bodies should be discarded as they could give rise to false results.
5. Sera and plasma can be stored at +2°...+8°C in primary collection tubes for up to five days after collection. Do not freeze primary tubes of collection. For longer storage periods, sera and plasma samples, carefully removed from the primary collection tube, can be stored frozen at -20°C for at least 12 months. Any frozen samples should not be frozen/thawed more than once as this may generate particles that could affect the test result.
6. If particles are present, centrifuge at 2,000 rpm for 20 min or filter using 0.2-0.8u filters to clean up the sample for testing.

H. PREPARATION OF COMPONENTS AND WARNINGS

A study conducted on an opened kit has not pointed out any relevant loss of activity up to 6 re-uses of the device and up to 3 months.

1. Microplate:
Allow the microplate to reach room temperature (about 1 hr) before opening the container. Check that the desiccant has not turned dark green, indicating a defect in manufacturing. In this case, call DiaPro's customer service.
Unused strips have to be placed back into the aluminum pouch, with the desiccant supplied, firmly zipped and stored at +2°-8°C. When opened the first time, unused strips are stable until the humidity indicator inside the desiccant bag turns from yellow to green.
2. Negative Control:
Ready to use. Mix well on vortex before use.
3. Antigen Positive Control:
Ready to use. Mix well on vortex before use.
4. Antibody Positive Control:
Ready to use. Mix well on vortex before use.
5. Antigen Calibrator:
Add the volume of ELISA grade water, reported on the label, to the lyophilized powder; let fully dissolve and then gently mix on vortex.

Note: The dissolved calibrator is not stable. Store it frozen in aliquots at -20°C.

6. Antibody Calibrator:
Add the volume of ELISA grade water, reported on the label, to the lyophilized powder; let fully dissolve and then gently mix on vortex.
Note: The dissolved calibrator is not stable. Store it frozen in aliquots at -20°C.

7. Wash buffer concentrate:
The whole content of the 20x concentrated solution has to be diluted with distilled water up to 1200 ml and mixed gently end-over-end before use.
During preparation avoid foaming as the presence of bubbles could impact on the efficiency of the washing cycles.
Note: Once diluted, the wash solution is stable for 1 week at +2...8° C.

8. Enzyme conjugate:
Ready to use. Mix well on vortex before use.
Avoid contamination of the liquid with oxidizing chemicals, air-driven dust or microbes. If this component has to be transferred, use only plastic, and if possible, sterile disposable containers.

9. HBe Antigen:
Ready to use. Mix well on vortex before use.
Avoid contamination of the liquid with oxidizing chemicals, air-driven dust or microbes. If this component has to be transferred, use only plastic, and if possible, sterile disposable containers.

10. Chromogen/Substrate:
Ready to use. Mix well on vortex before use.
Avoid contamination of the liquid with oxidizing chemicals, air-driven dust or microbes. Do not expose to strong light, oxidizing agents and metallic surfaces.
If this component has to be transferred use only plastic, and if possible, sterile disposable container.

11. Sulphuric Acid:
Ready to use. Mix well on vortex before use.
Attention: Infant (H315, H319; P280, P302+P352, P332+P313, P305+P351+P338, P337+P313, P362+P363).

Legenda:

Warning H statements:

- H315 – Causes skin irritation.
- H319 – Causes serious eye irritation.

Precautionary P statements:

- P280 – Wear protective gloves/protective clothing/eye protection/face protection.

- P302 + P352 – IF ON SKIN: Wash with plenty of soap and water.
- P332 + P313 – IF skin irritation occurs: Get medical advice/attention.
- P305 + P351 + P338 – IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
- P337 + P313 – IF eye irritation persists: Get medical advice/attention.
- P362 + P363 – Take off contaminated clothing and wash it before reuse.

L. PRE ASSAY CONTROLS AND OPERATIONS

1. Check the expiration date of the kit printed on the external label (primary container). Do not use if expired.
2. Check that the liquid components are not contaminated by visible particles or aggregates. Check that the Chromogen/Substrate (TMB+H₂O₂) is colourless or pale blue by aspirating a small volume of it with a sterile plastic pipette. Check that no leakage occurred in transportation

I. INSTRUMENTS AND TOOLS USED IN COMBINATION WITH THE KIT

1. Micropipettes have to be calibrated to deliver the correct volume required by the assay and must be submitted to regular decontamination (household alcohol, 10% solution of bleach, hospital grade disinfectants) of those parts that could accidentally come in contact with the sample. Decontamination of spills or residues of kit components should also be carried out regularly. They should also be regularly maintained in order to show a precision of 1% and a linearity of ±2%.

2. The ELISA incubator has to be set at +37°C (tolerance of ±0.5°C) and regularly checked to ensure the correct temperature is maintained. Both dry incubators and water baths are suitable for the incubations, provided that the instrument is validated for the incubation or ELISA tests.

3. The ELISA washer is extremely important to the overall performance of the assay. The washer must be carefully validated in advance, checked for the delivery of the right dispensation volume and regularly submitted to maintenance according to the manufacturer's instructions for use. In particular the washer, at the end of the daily workload, has to be extensively cleaned out of salts with deionized water. Before use, the washer has to be extensively primed with the diluted Washing Solution. The instrument weekly has to be submitted to decontamination according to its manual (NaOH 0.1 M decontamination suggested).

5. Washing cycles (aspiration + dispensation of 350ul/well of washing solution + 20 sec soaking = 1 cycle) are sufficient to ensure the assay with the declared performances. If soaking is not possible add one more cycle of washing.

An incorrect washing cycle or salt-blocked needles are the major cause of false positive reactions.

4. Incubation times have a tolerance of ±5%.
5. The ELISA reader has to be equipped with a reading filter of 450nm and with a second filter of 620-630nm, mandatory for the blanking purposes. Blanking is carried out on the well identified in the section "Assay Procedure". The optical system of the reader has to be calibrated regularly to ensure the correct optical density is measured. It should be regularly maintained according to the manufacturer's instructions.

6. When using an ELISA automated work station, all critical steps (dispensation, incubation, washing, reading, data handling) have to be carefully set, calibrated, controlled and regularly serviced in order to match the values reported in the section "Internal Quality Control". The assay protocol has to be installed in the operating system of the unit and validated as for the washer and the reader. In addition, the liquid handling part of the station (dispensation and washing) has to be validated and correctly set. Particular attention must be paid to avoid carry over by the needles used for dispensing and for washing. This must be studied and controlled to minimize the possibility of contamination of adjacent wells. The use of ELISA automated work stations is recommended when the number of samples to be tested exceed 20-30 units per run.

7. DiaPro's customer service offers support to the user in the setting and checking of instruments used in combination with the kit, in order to assure compliance with the requirements described. Support is also provided for the installation of new instruments to be used with the kit.

and no spillage of liquid is present inside the box (primary container). Check that the aluminium pouch, containing the microplate, is not punctured or damaged.

3. Dilute all the content of the 20x concentrated Wash Solution as described above.
4. Dissolve the Calibrator as described above and gently mix.
5. Allow all the other components to reach room temperature (about 1 hr) and then mix gently on vortex all liquid reagents.

6. Set the ELISA incubator at +37°C and prepare the ELISA washer by priming with the diluted washing solution, according to the manufacturers' instructions. Set the right number of washing cycles as reported in the specific section.
7. Check that the ELISA reader is turned on or ensure it will be turned on at least 20 minutes before reading.
8. If using an automated work station, turn on, check settings and be sure to use the right assay protocol.
9. Check that the microplate is set to the required volume.
10. Check that all the other equipment is available and ready to use.

In case of problems, do not proceed further with the test and advise the supervisor.

M. ASSAY PROCEDURE

The assay has to be performed according to the procedure given below, taking care to maintain the same incubation time for all the samples being tested.

A) HBe Antigen:

1. Place the required number of strips in the plastic holder and carefully identify the wells for controls, calibrator and samples.
2. Leave the A1 well empty for blanking purposes.
3. Pipette 100 µl of the Negative Control in triplicate, 100 µl of the Antigen Calibrator in duplicate and then 100 µl of the Antigen Positive Control in single.
4. Then dispense 100 µl of samples in the proper wells.
5. Check for the presence of samples in wells by naked eye (there is a marked colour difference between empty and full wells) or by reading at 450/620nm (samples show OD values higher than 0.100).
6. Incubate the microplate for 60 min at +37°C.

Important note: Strips have to be sealed with the adhesive sealing foil, only when the test is performed manually. Do not cover strips when using ELISA automatic instruments.

7. When the first incubation is finished, wash the microwells as previously described (section I.3)

8. Dispense 100 µl Enzyme Conjugate in all wells, except for A1, used for blanking operations.

Important note: Be careful not to touch the inner surface of the well with the pipette tip and not to immerse the top of it into samples or controls. Contamination might occur.

9. Check that the reagent has been dispensed properly and then incubate the microplate for 60 min at +37°C.
10. When the second incubation is finished, wash the microwells as previously described (section I.3)
11. Pipette 100 µl Chromogen/Substrate into all the wells, A1 included.

Important note: Do not expose to strong direct light as a high background might be generated.

12. Incubate the microplate protected from light at room temperature (18-24°C) for 20 minutes. Wells dispensed with positive control and positive samples will turn from clear to blue.

HBe antibody test

Controls and calibrator Samples	50 µl
Neutralising antigen	50 µl
1 st Incubation Temperature	60 min +37°C
Wash step	n° 5 cycles with 20° of soaking OR n° 6 cycles without soaking
Enzymatic conjugate	100 µl
2 nd Incubation Temperature	60 min +37°C
Wash step	n° 5 cycles with 20° of soaking OR n° 6 cycles without soaking
TMB/H2O2 mixture	100 µl
3 rd Incubation Temperature	20 min r.t.
Sulphuric Acid	100 µl
Reading OD	450nm/620-630nm

An example of dispensation scheme is reported below:

		Microplate											
		1	2	3	4	5	6	7	8	9	10	11	12
A	BLK	S2											
B	NC	S3											
C	NC	S4											
D	NC	S5											
E	CAL	S6											
F	CAL	S7											
G	PC	S8											
H	S1	S9											

Legend: BLK = Blank // NC = Negative Control // CAL = Calibrators // S = Sample

O. INTERNAL QUALITY CONTROL

A validation check is carried out on the controls any time the kit is used in order to verify whether the performances of the assay are as qualified.

Control that the following data are matched:

HBe Antigen	
Blank well	< 0.100 OD450nm
Negative Control (NC)	< 0.150 OD450nm after blanking coefficient of variation < 30%
Antigen Calibrator	SI/Co > 2.0
Positive Control (PC)	> 1.500 OD450nm

HBe Antibody	
Blank well	< 0.100 OD450nm
Negative Control (NC)	> 1.000 OD450nm after blanking coefficient of variation < 10%
Antibody Calibrator	OD450nm < NC/1.5
Positive Control (PC)	OD450nm < NC/10

If the results of the test match the requirements stated above, proceed to the next section.

If they do not, don't proceed any further and perform the following checks.

HBeAg

Problem	Check
Blank well > 0.100 OD450nm	1. that the Chromogen/Substrate solution has not become contaminated during the assay
Negative Control (NC) > 0.150 OD450nm after blanking coefficient of variation > 30%	1. that the washing procedure and the washer settings are as validated in the pre qualification study; 2. that the proper washing solution has been used and the washer has been primed with it before use; 3. that no mistake has been done in the assay procedure (dispensation of positive control instead of negative control); 4. that the control (dispensation of negative control) has occurred due to positive samples, to spills or to the enzyme conjugate; 5. that microplate has not become contaminated with positive samples or with the enzyme conjugate; 6. that the washer needles are not blocked or partially obstructed.
Calibrator SI/Co < 2	1. that the procedure has been correctly performed; 2. that no mistake has occurred during its distribution (ex.: dispensation of negative control instead); 3. that the washing procedure and the washer settings are as validated in the pre qualification study; 4. that no external contamination of the calibrator has occurred.
Positive Control < 1.500 OD450nm	1. that the procedure has been correctly performed; 2. that no mistake has occurred during the distribution of the control (dispensation of negative control instead of positive control); 3. that the washing procedure and the washer settings are as validated in the pre qualification study; 4. that no external contamination of the positive control has occurred.

HBe antibody

Problem	Check
Blank well > 0.100 OD450nm	1. that the Chromogen/Substrate solution has not become contaminated during the assay
Negative Control (NC) < 1.000 OD450nm after blanking coefficient of variation > 10%	1. that the washing procedure and the washer settings are as validated in the pre qualification study; 2. that the proper washing solution has been used and the washer has been primed with it before use; 3. that no mistake has been done in the assay procedure (e.g.: dispensation of positive control instead of negative control; no dispensation of the Neutralizing Antigen; no dispensation of the Enzyme Conjugate); 4. that no contamination of the negative control or of the wells where the control was dispensed has occurred; 5. that microplate has not become contaminated with positive samples; 6. that the washer needles are not blocked or partially obstructed.

Calibrator OD450nm > NC/1.5	1. that the procedure has been correctly performed;
	2. that no mistake has occurred during the distribution (ex.: dispensation) of negative control material; no dispersion of the Enzygnost Antigen; no dispensation of the Enzygnost Conjugate;
Positive Control OD450nm > NC/10	3. that the washing procedure and the washer settings are as validated in the pre qualification study;
	4. that no external contamination of the calibrator has occurred.
Positive Control OD450nm > NC/10	1. that the procedure has been correctly performed;
	2. that no mistake has occurred during the distribution of the control;
	3. that the washing procedure and the washer settings are as validated in the pre qualification study;
	4. that no external contamination of the positive control has occurred.

If any of the above problems have occurred, report the problem to the supervisor for further actions.

Important note:

The analysis must be done proceeding as the reading step described in the section M, point 14.

P. CALCULATION OF THE CUT-OFF

The results are calculated by means of a cut-off value determined with the following formula:

HBeAg:

$$NC + 0.100 = \text{Cut-Off (Co)}$$

The value found for the test is used for the interpretation of results as described in the next paragraph.

HBeAb:

$$(NC + PC) / 3 = \text{Cut-Off (Co)}$$

Important note: When the calculation of results is performed by the operating system of an ELISA automated work station, ensure that the proper formulation is used to calculate the cut-off value and generate the correct interpretation of results.

Q. INTERPRETATION OF RESULTS

Results are interpreted as follows:

HBeAg:

S/Co	Interpretation
<0.9	Negative
0.9-1.1	Equivocal
> 1.1	Positive

HBeAb:

Co/S	Interpretation
<0.9	Negative
0.9-1.1	Equivocal
> 1.1	Positive

Note:

S = OD450nm/620-630nm of the sample
Co = cut-off value

An example of calculation for HBeAg assay is reported below (data obtained proceeding as the the reading step described in the section M, point 14):

The following data must not be used instead of real figures obtained by the user:

Negative Control: 0.020 – 0.030 – 0.025 OD450nm
Mean Value: 0.025 OD450nm
Lower than 0.150 – Accepted

Positive Control: 2.489 OD450nm
Higher than 1.500 – Accepted
Cut-Off = 0.025+0.100 = 0.125

Calibrator: 0.520 - 0.540 OD450nm S/Co = 4.2
Mean value: 0.530 OD450nm
S/Co higher than 2.0 – Accepted

Sample 1: 0.030 OD450nm
Sample 2: 1.800 OD450nm
Sample 1 S/Co < 0.9 = negative
Sample 2 S/Co > 1.1 = positive

An example of calculation for HBeAb is reported below (data obtained proceeding as the the reading step described in the section M, point 14):

The following data must not be used instead of real figures obtained by the user.

Negative Control: 2.100 – 2.200 – 2.000 OD450nm
Mean Value: 2.100 OD450nm
Higher than 1.000 – Accepted

Positive Control: 0.100 OD450nm
Lower than NC/10 – Accepted

Cut-Off = (2.100 + 0.100) / 3 = 0.733
Calibrator: 0.720-0.760 OD450nm
Mean value: 0.740 OD450nm
OD450nm < NC/1.5 – Accepted

Sample 1: 0.020 OD450nm
Sample 2: 1.900 OD450nm
Sample 1 Co/S > 1.1 positive
Sample 2 Co/S < 0.9 negative

Important notes:

- Interpretation of results should be done under the supervision of the laboratory director to reduce the risk of judgment errors and misinterpretations.
- The identification of the clinical status of a HBV patient (acute, chronic, asymptomatic hepatitis) has to be done on the basis also of the other markers of HBV infection (HBSAg, HBSAb, HBeAg, HBeAb, HSCr);
- When test results are transmitted from the laboratory to another facility, attention must be paid to avoid erroneous data transfer.
- Diagnosis of viral hepatitis infection has to be taken by and released to the patient by a suitably qualified medical doctor.

11	1	3.4	3.6
12	<1	0.2	1.2
13	<1	0.9	1.4
14	-	0.2	0.2
15	-	0.4	0.1
16	-	0.5	0.1
17	-	0.3	0.2
18	-	0.2	0.2
19	-	0.2	0.1
20	-	0.2	0.1
21	-	0.3	1.0
22	-	0.3	0.1
23	-	0.4	0.1
24	-	0.2	0.2
25	-	0.3	0.2

3. Diagnostic Specificity:

The diagnostic specificity has been determined on panels of negative samples from normal individuals and blood donors, classified negative with a FDA approved kit.

Both plasma, derived with different standard techniques, of preparation (citrate, EDTA and heparin), and sera have been used to determine the specificity.
No false reactivity due to the method of specimen preparation has been observed.

Frozen specimens have also been tested to check whether this interferes with the performance of the test. No interference was observed on clean and particle free samples.

Samples derived from patients with different viral (HCV and HAV) and non viral pathologies of the liver that may interfere with the test were examined.

No cross reaction were observed.

The Performance Evaluation study conducted in a qualified external reference center on more than 500 samples has provided a value > 98%.

4. Precision

It has been calculated on two samples examined in 16 replicate in three different runs on three lots.
The values found were as follows:

HBE,CE: lot # 0103

Negative Control (N = 16)			
Mean values	1st run	2nd run	3rd run
OD 450nm	0.030	0.027	0.032
Std.Deviation	0.002	0.002	0.003
CV %	7.4	8.2	7.9

PEI 1 U/ml (N = 16)

Mean values			
	1st run	2nd run	3rd run
OD 450nm	0.569	0.559	0.575
Std.Deviation	0.027	0.029	0.028
CV %	4.7	5.3	4.9
S/Co	4.4	4.4	4.4

HBE,CE: lot # 01032

Negative Control (N = 16)			
Mean values	1st run	2nd run	3rd run
OD 450nm	0.033	0.031	0.030
Std.Deviation	0.003	0.003	0.002
CV %	7.9	8.5	7.4

PEI 1 U/ml (N = 16)

Mean values			
	1st run	2nd run	3rd run
OD 450nm	0.565	0.573	0.568
Std.Deviation	0.026	0.025	0.024
CV %	4.7	4.3	4.2
S/Co	4.2	4.4	4.4

HBE CE: lot # 0303

Negative Control (N = 16)				
Mean values	1st run	2nd run	3rd run	Average value
OD 450nm	0.029	0.034	0.038	0.034
Std Deviation	0.003	0.003	0.004	0.003
CV %	9.7	9.8	9.2	9.6

PEI 1 U/ml (N = 16)				
Mean values	1st run	2nd run	3rd run	Average value
OD 450nm	0.579	0.573	0.564	0.572
Std Deviation	0.023	0.029	0.025	0.025
CV %	4.1	4.8	4.5	4.5
CoS	4.5	4.3	4.1	4.3

B) HBe Antibody

1. Limit of detection

The limit of detection of the assay has been calculated by means of the International Standard for HBeAb, supplied by Paul Ehrlich Institute (PEI).

The data obtained by examining the limit of detection on three lots is reported in the table below.

HBE CE	PEI U/ml
Lot ID	HBeAb
0103	0.25
0103/2	0.25
0303	0.25

In addition the preparation Accurun # 52, produced by Boston Biomedica Inc., USA, has been tested, upon dilution in FCS. Results are reported for three lots of products.

Accurun 52 (CoS)				
HBE CE	1 x	2 x	4 x	8 x
Lot ID	1.0	0.8	0.6	0.4
0103	1.0	0.8	0.6	0.4
0103/2	1.0	0.8	0.6	0.4
0303	1.0	0.8	0.6	0.4

2. Diagnostic sensitivity

The diagnostic sensitivity has been tested on panels of samples classified positive for HBeAb by a US FDA approved kit. Positive samples were collected from different HBV pathologies bearing anti HBeAg antibody reactivity.

An overall value > 98% has been found in the study conducted on a total number of more than 200 samples.

Moreover the Panel of Seroconversion code PHM 935B, produced by BBI, was examined.

Data are reported below and compared with those reported by BBI for two other commercial products.

Sample ID	HBE CE	Abbott EIA	Serip EIA
CoS	CoS	CoS	CoS
21	0.4	0.5	0.5
22	0.4	0.5	0.6
23	0.4	0.5	0.5
24	0.4	0.5	0.6
25	0.4	0.6	0.5
26	0.5	0.6	0.6
27	0.6	0.6	0.7
28	0.7	0.9	0.7
29	0.6	0.9	0.7
30	0.8	1.0	0.9
31	1.0	1.3	1.1
32	1.0	1.2	1.0

Finally the Performance Panel code PHU 201, produced by BBI, was tested. Data are reported below and compared with those reported by BBI for another commercial product.

Number	PEI U/ml	HBE CE	Serip EIA
1	-	0.3	0.5
2	-	0.2	0.5
3	-	0.2	0.5
4	-	0.2	0.5
5	-	0.3	0.8
6	-	0.3	0.8
7	-	0.2	0.4
8	-	0.2	0.5
9	-	0.2	0.5
10	-	1.9	0.6
11	-	0.3	0.5
12	-	0.4	0.8
13	-	0.4	0.8
14	-	4.4	9.1
15	-	3.8	2.9
16	> 50	4.3	120.9
17	< 1	1.0	1.5
18	< 1	1.0	1.0
19	5	5.6	21.8
20	1	2.7	6.4
21	2	1.9	10.0
22	26	28.1	90.7
23	< 1	0.3	0.5
24	< 1	0.8	1.3
25	50	28.1	167.4

3. Diagnostic specificity

The clinical specificity has been determined as described before for HBeAg.

The Performance Evaluation study conducted in a qualified external reference center on more than 500 samples has provided a value > 98%.

4. Precision

It has been calculated on two samples examined in 16 replicate in three different runs on three lots.

The values found were as follows:

HBE CE: lot # 0103

Negative Control (N = 16)				
Mean values	1st run	2nd run	3rd run	Average value
OD 450nm	2.484	2.420	2.471	2.459
Std Deviation	0.129	0.150	0.142	0.144
CV %	5.2	6.6	5.7	5.9

PEI 0.25 U/ml (N = 16)				
Mean values	1st run	2nd run	3rd run	Average value
OD 450nm	0.857	0.800	0.878	0.848
Std Deviation	0.043	0.060	0.050	0.051
CV %	5.0	7.5	5.7	6.1
CoS	1.0	1.0	1.0	1.0

HBE CE: lot # 0103/2

Negative Control (N = 16)				
Mean values	1st run	2nd run	3rd run	Average value
OD 450nm	2.316	2.361	2.413	2.363
Std Deviation	0.127	0.144	0.146	0.139
CV %	5.5	6.1	6.0	5.9

PEI 0.25 U/ml (N = 16)

Mean values	1st run	2nd run	3rd run	Average value
OD 450nm	0.767	0.793	0.785	0.781
Std Deviation	0.041	0.050	0.046	0.046
CV %	5.4	6.3	5.8	5.8
CoS	1.0	1.0	1.0	1.0

HBE CE: lot #0303

Negative Control (N = 16)

Mean values	1st run	2nd run	3rd run	Average value
OD 450nm	2.334	2.415	2.437	2.395
Std Deviation	0.146	0.155	0.159	0.153
CV %	6.3	6.4	6.5	6.4

PEI 0.25 U/ml (N = 16)

Mean values	1st run	2nd run	3rd run	Average value
OD 450nm	0.850	0.867	0.878	0.864
Std Deviation	0.052	0.051	0.048	0.050
CV %	6.1	5.9	5.5	5.8
CoS	0.9	1.0	1.0	1.0

Important note:
The performance data have been obtained proceeding as the reading step described in the section M, point 14.

5. LIMITATIONS

Frozen samples containing fibrin particles or aggregates may generate false positive results.

Bacterial contamination or heat inactivation of the specimen may affect the absorbance values of the samples with consequent alteration of the level of the analyte.

This test is suitable only for testing single samples and not pooled ones.

Diagnosis of an infectious disease should not be established on the basis of a single test result. The patient's clinical history, symptomatology, as well as other diagnostic data should be considered.

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All the IVD Products manufactured by the company are under the control of a certified Quality Management System approved by an EC Notified Body. Each lot is submitted to a quality control and released into the market only if conforming with the EC technical specifications and acceptance criteria.

Manufacturer:

Dia.Pro Diagnostic Bioprobes S.r.l.
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0318

HCV Ab

Version 4.0 Enzyme Immunoassay for the determination of anti Hepatitis C Virus antibody in human serum and plasma

- for "in vitro" diagnostic use only -



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REF CVAB/CE
96,192,480,960 Tests

HCV Ab

A. INTENDED USE

Version 4.0 Enzyme ImmunoAssay (ELISA) for the determination of antibodies to Hepatitis C Virus in human plasma and sera. The kit is intended for the screening of blood units and the follow-up of HCV-infected patients. For "in vitro" diagnostic use only.

B. INTRODUCTION

The World Health Organization (WHO) define Hepatitis C infection as follows:

"Hepatitis C is a viral infection of the liver which had been referred to as parenterally transmitted "non A, non B hepatitis" until identification of the causative agent in 1989. The discovery and characterization of the hepatitis C virus (HCV) led to the understanding of its primary role in post-transfusion hepatitis and its tendency to induce persistent infection.

HCV is a major cause of acute hepatitis and chronic liver disease, including cirrhosis and liver cancer. Globally, an estimated 170 million persons are chronically infected with HCV and 3 to 4 million persons are newly infected each year. HCV is spread primarily by direct contact with human blood. The major causes of HCV infection worldwide are use of unscreened blood transfusions, and reuse of needles and syringes that have not been adequately sterilized. No vaccine is currently available to prevent hepatitis C and treatment for chronic hepatitis C is too costly for most persons in developing countries to afford. Thus, from a global perspective, the greatest impact on hepatitis C disease burden will likely be achieved by focusing efforts on reducing the risk of HCV transmission from nosocomial exposures (e.g. blood transfusions, unsafe injection practices) and high-risk behaviours (e.g. injection drug use).

Hepatitis C virus (HCV) is one of the viruses (A, B, C, D, and E), which together account for the vast majority of cases of viral hepatitis. It is an enveloped RNA virus in the *flaviviridae* family which appears to have a narrow host range. Humans and chimpanzees are the only known species susceptible to infection, with both species developing similar disease.

An important feature of the virus is the relative instability of its genome, which in turn is probably related to the high propensity (80%) of inducing chronic infection. HCV is clustered into several distinct genotypes which may be important in determining the severity of the disease and the response to treatment.

The incubation period of HCV infection before the onset of clinical symptoms ranges from 15 to 150 days. In acute infections, the most common symptoms are fatigue and jaundice; however, the majority of cases between 60% and 70%, even those that develop chronic infection, are asymptomatic. About 80% of newly infected patients progress to develop chronic infection. Cirrhosis develops in about 10% to 20% of persons with chronic infection, and liver cancer develops in 1% to 5% of persons with chronic infection over a period of 20 to 30 years. Most patients suffering from liver cancer who do not have hepatitis B virus infection have evidence of HCV infection. The mechanisms by which HCV infection leads to liver cancer are still unclear. Hepatitis C also exacerbates the severity of underlying liver disease when it coexists with other hepatic conditions. In particular, liver disease progresses more rapidly among persons with

alcoholic liver disease and HCV infection. HCV is spread primarily by direct contact with human blood. Transmission through blood transfusions that are not screened for HCV infection, through the reuse of inadequately sterilized needles, syringes or other medical equipment, or through needle-sharing among drug-users, is well documented. Sexual and perinatal transmission may also occur although less frequently. Other modes of transmission such as social, cultural, and behavioural practices using percutaneous procedures (e.g. ear and body piercing, circumcision, tattooing) can occur if inadequately sterilized equipment is used. HCV is not spread by sneezing, hugging, coughing, food or water, sharing eating utensils, or casual contact.

In both developed and developing countries, high risk groups include injecting drug users, recipients of unscreened blood, haemophiliacs, dialysis patients and persons with multiple sex partners who engage in unprotected sex. In developed countries, it is estimated that 90% of persons with chronic HCV infection are current and former injecting drug users and those with a history of transfusion of unscreened blood or blood products. In many developing countries, where unscreened blood and blood products are still being used, the major means of transmission are unscreened injection, equipment and unscreened blood transfusions. In addition, people who use traditional scarification and circumcision practices are at risk if they use or re-use unsanitized tools.

WHO estimates that about 170 million people, 3% of the world's population, are infected with HCV and are at risk of developing liver cirrhosis and/or liver cancer. The prevalence of HCV infection in some countries in Africa, the Eastern Mediterranean, South-East Asia and the Western Pacific (when prevalence data are available) is high compared to some countries in North America and Europe.

Diagnostic tests for HCV are used to prevent infection through screening of donor blood and plasma, to establish the clinical diagnosis and to make better decisions regarding medical management of a patient. Diagnostic tests commercially available today are based on Enzyme immunoassay (EIA) for the detection of HCV specific antibodies. EIAs can detect more than 95% of chronically infected patients but can detect only 50% to 70% of acute infections. A recombinant immunoblot assay (RIBA) that identifies antibodies which react with individual HCV antigens is often used as a supplemental test for confirmation of a positive EIA result. Testing for HCV circulating by amplification tests RNA (e.g. polymerase chain reaction or PCR, branched DNA assay) is also being utilized for confirmation of serological results as well as for assessing the effectiveness of antiviral therapy. A positive result indicates the presence of active infection and a potential for spread of the infection and/or the development of chronic liver disease.

Antiviral drugs such as interferon taken alone or in combination with ribavirin, can be used for the treatment of persons with chronic hepatitis C, but the cost of treatment is very high. Treatment with interferon alone is effective in about 10% to 20% of patients. Interferon combined with ribavirin is effective in about 30% to 50% of patients. Ribavirin does not appear to be effective when used alone.

There is no vaccine against HCV. Research is in progress but the high mutability of the HCV genome complicates vaccine development. Lack of knowledge of any protective immune response following HCV infection also impedes vaccine research. It is not known whether the immune system is able to eliminate the virus.

Some studies, however, have shown the presence of virus neutralizing antibodies in patients with HCV infection. In the absence of a vaccine, all precautions to prevent infection must be taken including (a) screening and testing of blood and organ donors; (b) Virus inactivation of plasma derived products; (c) implementation and maintenance of infection control practices in health care settings including appropriate sterilization of medical and dental equipment; (d) promotion of behaviour change among the general public and health care workers to reduce sources of infections and to use safe injection practices; and (e) Risk reduction counselling for persons with high-risk drug and sexual practices."

The genome encodes for structural components, a nucleocapsid protein and two envelope glycoproteins, and functional constituents involved in the virus replication and protein processing. The nucleocapsid-encoding region seems to be the most conservative among the isolates obtained all over the world.

C. PRINCIPLE OF THE TEST

Microplates are coated with HCV-specific antigens derived from "core" and "ns" regions encoding for conservative and immunodominant antigenic determinants (Core peptide, recombinant NS3, NS4 and NS5 peptides).

The solid phase is first treated with the diluted sample and HCV Ab are captured, if present, by the antigens.

After washing out all the other components of the sample, in the 2nd step, peroxidase-conjugated anti-HCV antibodies are added. A cut-off value let optical densities be interpreted into HCV antibody negative and positive results.

D. COMPONENTS

Code CVAB/CE contains reagents for 192 tests.

1. Microplate MICROPLATE

n° 2 microplates

12 strips of 8 microwells coated with Core peptide, recombinant NS3, NS4 and NS5 peptides. Plates are sealed into a bag with desiccant.

2. Negative Control CONTROL

1x4.0ml/Vial. Ready to use control. It contains 1% goat serum proteins, 10 mM Na-citrate buffer pH 6.0 +/-0.1, 0.5% Tween 20, 0.09% Na-azide and 0.1% Kathon GC as preservatives. The negative control is olive green colour coded.

3. Positive Control CONTROL +

1x4.0ml/Vial. Ready to use control. It contains 1% goat serum proteins, human antibodies positive to HCV, 10 mM Na-citrate buffer pH 6.0 +/-0.1, 0.5% Tween 20, 0.09% Na-azide and 0.1% Kathon GC as preservatives. The Positive Control is blue colour coded.

4. Calibrator CAL

n° 2 vials. Lyophilized calibrator. To be dissolved with the volume of EIA grade water reported on the label. It contains foetal bovine serum proteins, human antibodies to HCV whose content is calibrated on the NIBSC Working Standard code 99/588-033-WI, 10 mM Na-citrate buffer pH 6.0 +/-0.1, 0.3 mg/ml gentamicine sulphate and 0.1% Kathon GC as preservatives.

Note: The volume necessary to dissolve the content of the vial may vary from lot to lot. Please use the right volume reported on the label.

5. Wash buffer concentrate WASHBUF 20X

2x60ml/bottle. 20x concentrated solution. Once diluted, the wash solution contains 10 mM phosphate buffer pH 7.0 +/-0.2, 0.05% Tween 20 and 0.05% Kathon GC.

6. Enzyme Conjugate CON

2x100ml/Vial. Ready to use and prepacked colour coded reagent. It contains Horseradish Peroxidase conjugated goat polyclonal antibodies to human IgG and IgM, 5% BSA, 10 mM Tris buffer pH 6.8 +/-0.1, 0.1% Kathon GC and 0.02% gentamicine sulphate as preservatives.

7. Chromogen/Substrate SUBS TMB

2x150ml/Vial. Ready-to-use component. It contains 50 mM citrate-phosphate buffer pH 3.5-3.8, 4% dimethylsulphoxide, 0.03% tetra-methyl-benzidine or TMB and 0.02% hydrogen peroxide or H₂O₂.

Note: To be stored protected from light as sensitive to strong illumination.

8. Assay Diluent DILAS

1x150ml/Vial. 10 mM Tris buffered solution pH 8.0 +/-0.1 containing 0.1% Kathon GC for the pre-treatment of samples and controls in the plate, blocking interference.

9. Sulphuric Acid H₂SO₄ 0.3 M

1x320ml/bottle. It contains 0.3 M H₂SO₄ solution. Attention: Irritant (H315; H319; P280; P302+P352; P332+P313; P305+P351+P338; P337+P313; P362+P363)

10. Sample Diluent DILSPE

2x50ml/bottle. It contains 1% goat serum proteins, 10 mM Na-citrate buffer pH 6.0 +/-0.1, 0.5% Tween 20, 0.09% Na-azide and 0.1% Kathon GC as preservatives. To be used to dilute the sample.

Note: The diluent changes colour from olive green to dark bluish green in the presence of sample.

11. Plate sealing foils n° 4

12. Package Insert n° 1

Important note: Only upon specific request, Dia.Pro can supply reagents for 96, 480, 960 tests, as reported below:

	n°1	n°5	n°10
1. Microplate	1x2.0ml/vial	1x10ml/vial	1x20 ml/vial
2. Negative Control	1x2.0ml/vial	1x10ml/vial	1x20 ml/vial
3. Positive Control	n° 1 vial	n° 5 vials	n° 10 vials
4. Calibrator	1x60ml/bottle	5x60ml/bottles	4x150ml/bottles
5. Wash buff conc	1x160ml/Vial	2x40ml/bottles	4x40ml/bottles
6. Enz. Conjugate	1x160ml/Vial	2x40ml/bottles	4x40ml/bottles
7. Chromogen/Subs	1x160ml/Vial	2x40ml/bottles	4x40ml/bottles
8. Assay Diluent	1x160ml/Vial	2x40ml/bottle	1x80ml/bottle
9. Sulphuric Acid	1x150ml/Vial	5x50ml/bottles	4x125ml/bottles
10. Sample Diluent	n° 2	n° 10	n° 20
11. Plate seal foils	n° 1	n° 1	n° 1
12. Pack. insert			
Number of tests	96	480	960
Code	CVAB/CE 96	CVAB/CE 480	CVAB/CE 960

E. MATERIALS REQUIRED BUT NOT PROVIDED

1. Calibrated Micropipettes (200ul and 10ul) and disposable plastic tips.
2. EIA grade water (distilled or deionised, charcoal treated to remove oxidizing chemicals used as disinfectants).
3. Timer with 60 minute range or higher.
4. Absorbent paper tissues.
5. Calibrated ELISA microplate thermostatic incubator capable to provide a temperature of +37°C.
6. Calibrated ELISA microwell reader with 450nm (reading) and with 620-630nm (blanking) filters.
7. Calibrated ELISA micropipette washer.
8. Vortex or similar mixing tools.

before waste. Suggested procedures of inactivation are treatment with a 10% final concentration of household bleach for 16-18 hrs or heat inactivation by autoclave at 121°C for 20 min.

15. Accidental spills from samples and operations have to be adsorbed with paper tissues soaked with household bleach and then with water. Tissues should then be discarded in proper containers designated for laboratory/hospital waste.

16. The Sulphuric Acid is an irritant. In case of spills, wash the surface with plenty of water.

17. Other waste materials generated from the use of the kit (example: tips used for samples and controls, used micropipettes) should be handled as potentially infective and disposed according to national directives and laws concerning laboratory wastes.

F. WARNINGS AND PRECAUTIONS

1. The kit has to be used by skilled and properly trained technical personnel only, under the supervision of a medical doctor responsible of the laboratory.

2. When the kit is used for the screening of blood units and blood components, it has to be used in a laboratory certified and qualified by the national authority in that field (Ministry of Health or similar entity) to carry out this type of analysis.

3. All the personnel involved in performing the assay have to wear protective laboratory clothes, lab-free gloves and glasses. The use of any sharp (needles) or cutting (blades) devices should be avoided. All the personnel involved should be trained in biosafety procedures, as recommended by the Center for Disease Control, Atlanta, U.S. and reported in the National Institute of Health's publication: "Biosafety in Microbiological and Biomedical Laboratories", ed. 1984.

4. All the personnel involved in sample handling should be vaccinated for HBV and HAV, for which vaccines are available, safe and effective.

5. The laboratory environment should be controlled so to avoid contaminants such as dust or air-borne microbial agents, when opening kit vials and microplates and when performing the test. Protect the Chromogen/Substrate from strong light and avoid vibration of the bench surface where the test is undertaken.

6. Upon receipt, store the kit at 2.8°C into a temperature controlled refrigerator or cold room.

7. Do not interchange components between different lots of the kits. It is recommended that components between two kits of the same lot should not be interchanged.

8. Check that the reagents are clear and do not contain visible heavy particles or aggregates. If not, advise the laboratory supervisor to initiate the necessary procedures for kit replacement.

9. Avoid cross-contamination between serum/plasma samples by using disposable tips and changing them after each sample.

10. Avoid cross-contamination between kit reagents by using disposable tips and changing them between the use of each one.

11. Do not use the kit after the expiration date stated on the external container and internal (vials) labels.

12. Treat all specimens as potentially infective. All human serum specimens should be handled at Biosafety Level 2, as recommended by the Center for Disease Control, Atlanta, U.S. in compliance with what reported in the Institutes of Health's publication: "Biosafety in Microbiological and Biomedical Laboratories", ed. 1984.

13. The use of disposable plastic-ware is recommended in the preparation of the liquid components or in transferring components into automated workstations, in order to avoid cross contamination.

14. Waste produced during the use of the kit has to be discarded in compliance with national directives and laws concerning laboratory waste of chemical and biological substances. In particular, liquid waste generated from the washing procedure, from residues of controls and from samples has to be treated as potentially infective material and inactivated

H. PREPARATION OF COMPONENTS AND WARNINGS

A study conducted on an opened kit has not pointed out any relevant loss of activity up to 6 re-use of the device and up to 6 months.

1. Microplates:

Allow the microplate to reach room temperature (about 1 hr) before opening the container. Check that the desiccant is not turned to dark green, indicating a defect of manufacturing. In this case call Dia.Pro's customer service.

Unused strips have to be placed back into the aluminium pouch, in presence of desiccant, supplied, firmly zipped and stored at +2°-8°C.

When opened the first time, residual strips are stable till the indicator of humidity inside the desiccant bag turns from yellow to green.

2. Negative Control:

Ready to use. Mix well on vortex before use.

3. Positive Control:

Ready to use. Mix well on vortex before use. Handle this component as potentially infective, even if HCV, eventually present in the control, has been chemically inactivated.

4. Calibrator:

Dissolve carefully the content of the lyophilised vial with the volume of EIA grade water reported on its label. Mix well on vortex before use.

Handle this component as potentially infective, even if HCV, eventually present in the control, has been chemically inactivated.

Note: When dissolved the Calibrator is not stable. Store in aliquots at -20°C.

5. Wash buffer concentration:

The 20x concentrated solution has to be diluted with EIA grade water-up to 1200 ml and mixed gently end-over-end before use. As some salt crystals may be present into the vial, take care to dissolve all the content when preparing the solution.

In the preparation avoid foaming as the presence of bubbles could give origin to a bad washing efficiency.

Note: Once diluted, the wash solution is stable for 1 week at +2,8°C.

6. Enzyme conjugate:

Ready to use. Mix well on vortex before use.

Be careful not to contaminate the liquid with oxidizing chemicals, air-driven dust or microbes.

If this component has to be transferred use only plastic, possibly sterile disposable containers.

7. Chromogen/Substrate:

Ready to use. Mix well on vortex before use.

Be careful not to contaminate the liquid with oxidizing chemicals, air-driven dust or microbes.

Do not expose to strong illumination, oxidizing agents and metallic surfaces.

If this component has to be transferred use only plastic, possible sterile disposable container.

8. Assay Diluent:

Ready to use. Mix well on vortex before use.

9. Sulphuric Acid:

Ready to use. Mix well on vortex before use.

Attention: Irritant (H315; H319; P280; P302+P352; P332+P313; P305+P351+P338; P337+P313; P362+P363).

Precautionary P statements:

P280 – Wear protective gloves/protective clothing/eye protection/face protection.

P302 + P352 – IF ON SKIN: Wash with plenty of soap and water.

P332 + P313 – IF skin irritation occurs: Get medical advice/attention.

P305 + P351 + P338 – IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do.

P337 + P313 – If eye irritation persists: Get medical advice/attention.

P362 + P363 – Take off contaminated clothing and wash it before reuse.

10. Sample Diluent:

Ready to use. Mix well on vortex before use.

L. INSTRUMENTS AND TOOLS USED IN COMBINATION WITH THE KIT

1. Micropipettes have to be calibrated to deliver the correct volume required by the assay and must be submitted to regular decontamination (household alcohol; 10% solution of bleach, hospital grade disinfectants) of those parts that could accidentally come in contact with the sample. They should also be regularly maintained in order to show a precision of 1% and a trueness of +/-2%. Decontamination of spills or residues of kit components should also be carried out regularly.

2. The ELISA incubator has to be set at +37°C (tolerance of +/-0,5°C) and regularly checked to ensure the correct temperature is maintained. Both dry incubators and water baths are suitable for the incubations, provided that the instrument is validated for the incubation of ELISA tests.

3. The ELISA washer is extremely important to the overall performances of the assay. The washer must be carefully

validated and correctly optimized using the kit controls and reference panels, before using the kit for routine laboratory tests. Usually 4-5 washing cycles (aspiration + dispensation of 350ul/well of washing solution = 1 cycle) are sufficient to ensure that the assay performs as expected. A soaking time of 20-30 seconds between cycles is suggested. In order to set correctly the number, it is recommended to run an assay with the kit controls and well characterized negative and positive reference samples, and check to match the values reported below in the sections "Validation of Test" and "Assay Performances". Regular calibration of the volumes delivered by, and maintenance (decontamination and cleaning of needles) of the washer has to be carried out according to the instructions of the manufacturer.

4. Incubation times have a tolerance of +/-5%.

5. The ELISA microplate reader has to be equipped with a reading filter of 450nm and with a second filter (620-630nm, strongly recommended) for blanking purposes. Its standard performances should be: (a) bandwidth ≤ 10 nm; (b) absorbance range from 0 to ≥ 2,0; (c) linearity to ≥ 2,0; (d) repeatability ≥ 1%. Blanking is carried out on the well identified in the section "Assay Procedure". The optical system of the reader has to be calibrated regularly to ensure that the correct optical density is measured. It should be regularly maintained according to the manufacturer's instructions.

6. When using an ELISA automated work station, all critical steps (dispensation, incubation, washing, reading, data handling) have to be carefully set, calibrated, controlled and regularly serviced in order to match the values reported in the section O "Internal Quality Control". The assay protocol has to be installed in the operating system of the unit and validated as for the washer and the reader. In addition, the liquid handling part of the station (dispensation and washing) has to be validated and correctly set. Particular attention must be paid to avoid carry over by the needles used for dispensing and for washing. This must be studied and controlled to minimize the possibility of contamination of adjacent wells. The use of ELISA automated work stations is recommended for blood screening when the number of samples to be tested exceed 20-30 units per run.

7. When using automatic devices, in case the val holder of the instrument does not fit with the vials supplied in the kit, transfer the solution into appropriate containers and label them with the same label peeled out from the original vial. This operation is important in order to avoid mismatching contents of vials, when transferring them. When the test is over, return the secondary labelled containers to 2,8°C, firmly capped.

8. Dia.Pro's customer service offers support to the user in the setting and checking of instruments used in combination with the kit, in order to assure compliance with the requirements described. Support is also provided for the installation of new instruments to be used with the kit.

L. PRE ASSAY CONTROLS AND OPERATIONS

1. Check the expiration date of the kit printed on the external label of the kit box. Do not use if expired.

2. Check that the liquid components are not contaminated by naked-eye visible particles or aggregates. Check that the Chromogen/Substrate is colorless or pale blue by aspirating a small volume of it with a sterile transparent plastic pipette. Check that no breakage occurred in transportation and no spillage of liquid is present inside the box. Check that the aluminum pouch, containing the microplate, is not punctured or damaged.

3. Dilute all the content of the 20x concentrated Wash Solution as described above.

4. Dissolve the Calibrator as described above.
5. Allow all the other components to reach room temperature (about 1 hr) and then mix as described.

6. Set the ELISA incubator at +37°C and prepare the ELISA washer by priming with the diluted washing solution, according to the manufacturers instructions. Set the right number of washing cycles as found in the validation of the instrument for its use with the kit.

7. Check that the ELISA reader has been turned on at least 20 minutes before reading.

8. If using an automated workstation, turn it on, check settings and be sure to use the right assay protocol.

9. Check that the micropipettes are set to the required volume.
10. Check that "all the other equipment" is available and ready to use.

11. In case of problems, do not proceed further with the test and advise the supervisor.

M. ASSAY PROCEDURE

The assay has to be carried out according to what reported below, taking care to maintain the same incubation time for all the samples in testing.

Automated assay:

In case the test is carried out automatically with an ELISA system, we suggest to make the instrument aspirate 200 ul Sample Diluent and then 10 ul sample.

All the mixture is then carefully dispensed directly into the appropriate sample well of the microplate. Before the next sample is aspirated, needles have to be duly washed to avoid any cross-contamination among samples.

Do not dilute controls/calibrator as they are ready to use.

Dispense 200 ul controls/calibrator in the appropriate control/calibrator wells.

Important Note: Visually monitor that samples have been diluted and dispensed into appropriate wells. This is simply achieved by checking that the colour of dispensed samples has turned to dark bluish-green while the colour of the negative control has remained olive green.

For the next operations follow the operative instructions reported below for the Manual Assay.

It is strongly recommended to check that the time lap between the dispensation of the first and the last sample will be calculated by the instrument and taken into consideration by delaying the first washing operation accordingly.

Manual assay:

1. Place the required number of Microwells in the microwell holder. Leave the 1st well empty for the operation of blanking.

2. Dispense 200 ul of Negative Control in triplicate, 200 ul Calibrator in duplicate and 200 ul Positive Control in single in proper wells. Do not dilute Controls and Calibrator as they are pre-diluted, ready to use!

3. Add 200 ul of Sample Diluent (DILSPE) to all the sample wells; then dispense 10 ul sample in each properly identified well. Mix gently the plate, avoiding overflowing and contaminating adjacent wells, in order to fully dispense the sample into its diluent.

Important note: Check that the colour of the Sample Diluent, upon addition of the sample, changes from light green to dark bluish green, monitoring that the sample has been really added.

4. Dispense 50 ul Assay Diluent (DILAS) into all the controls/calibrator and sample wells. Check that the color of samples has turned to dark blue.

5. Incubate the microplate for 45 min at +37°C.

Important note: Strips have to be sealed with the adhesive sealing foil, supplied only when the test is carried out manually. Do not cover strips when using ELISA automatic instruments.

6. Wash the microplate with an automatic washer by delivering and aspirating 350ul/well of diluted washing solution as reported previously (section L.3).
7. Pipette 100ul Enzyme Conjugate into each well, except the 1st blanking well, and cover with the sealer. Check that this pink/red coloured component has been dispensed in all the wells, except A1.

Important note: Be careful not to touch the plastic inner surface of the well with the tip filled with the Enzyme Conjugate. Contamination might occur.

8. Incubate the microplate for 45 min at +37°C.

9. Wash microwells as in step 6.

10. Pipette 100ul Chromogen/Substrate mixture into each well, the blank well included. Then incubate the microplate at room temperature (18-24°C) for 15 minutes.

Important note: Do not expose to strong direct illumination. High background might be generated.

11. Pipette 100ul Sulphuric Acid into all the wells using the same pipetting sequence as in step 10 to stop the enzymatic reaction. Addition of acid will turn the positive control and positive samples from blue to yellow/brown.

12. Measure the colour intensity of the solution in each well, as described in section L.5, at 450nm filter (reading) and at 620-630nm (background subtraction, strongly recommended), blanking the instrument on A1.

Important notes:

1. If the second filter is not available ensure that no finger prints are present on the bottom of the microwell before reading at 450nm. Finger prints could generate false positive results on reading.

2. Reading has to be carried out just after the addition of the Stop Solution and anyway not any longer than 20 minutes after its addition. Some self oxidation of the chromogen can occur leading to high background.

3. Shaking at 350 ±150 rpm during incubation has been proved to increase the sensitivity of the assay of about 20%.

4. The Calibrator (CAL) does not affect the cut-off calculation and therefore the test results calculation. The Calibrator may be used only when a laboratory internal quality control is required by the management.

N. ASSAY SCHEME

Method	Operations
Controls & Calibrator Samples	200 ul 200ul dil +10ul
Assay Diluent (DILAS)	50 ul
1 st incubation	45 min
Temperature	+37°C
Wash step	4-5 cycles
Enzyme conjugate	100 ul
2 nd incubation	45 min
Temperature	+37°C
Wash step	4-5 cycles
TMB/H2O2	100 ul
3 rd incubation	15 min
Temperature	r.t.
Sulphuric Acid	100 ul
Reading OD	450nm

An example of dispensation scheme is reported below:

Should these problems happen, after checking, report any residual problem to the supervisor for further actions.

Microplate											
	1	2	3	4	5	6	7	8	9	10	11
A	BLK	S2									
B	NC	S3									
C	NC	S4									
D	NC	S5									
E	CAL	S6									
F	CAL	S7									
G	PC	S8									
H	PC	S9									

BLK = Blank
CAL = Calibrator PC = Positive Control S = Sample

O. INTERNAL QUALITY CONTROL

A check is carried out on the controls and the calibrator any time the kit is used in order to verify whether their OD450nm values are as expected and reported in the table below.

Check	Requirements
Blank well	< 0.100 OD450nm value
Negative Control (NC)	< 0.050 mean OD450nm value after blanking
Calibrator	S/Co > 1.1
Positive Control	> 1.000 OD450nm value

If the results of the test match the requirements stated above, proceed to the next section.
If they do not, do not proceed any further and operate as follows:

Problem	Check
Blank well > 0.100 OD450nm	1. that the Chromogen/Substrate solution has not got contaminated during the assay
Negative Control (NC) > 0.050 OD450nm after blanking	1. that the washing procedure and the washer settings are as validated in the pre qualification study; 2. that the proper washing solution has been used and the washer has been primed with it 3. that no mistake has been done in the assay procedure (dispensation of positive control instead of negative control; 4. that no contamination of the negative control or of their wells has occurred due to positive samples, to spills or to the enzyme conjugate; 5. that micropipettes haven't got contaminated with positive samples or with the enzyme conjugate 6. that the washer needles are not blocked or partially obstructed
Calibrator S/Co < 1.1	1. that the procedure has been correctly executed; 2. that no mistake has been done in its dispensation (dispensation of negative control instead of control serum) 3. that the washing procedure and the washer settings are as validated in the pre qualification study; 4. that no external contamination of the calibrator has occurred
Positive Control < 1.000 OD450nm	1. that the procedure has been correctly executed; 2. that no mistake has been done in the distribution of controls (dispensation of negative control instead of positive control. In this case, the negative control will have an OD450nm value > 0.150, too. 3. that the washing procedure and its washer settings are as validated in the pre qualification study; 4. that no external contamination of the positive control has occurred

An example of calculation is reported below:

The following data must not be used instead of real figures obtained by the user.

Negative Control: 0.019 - 0.020 - 0.021 OD450nm

Mean Value: 0.020 OD450nm

Lower than 0.050 - Accepted

Positive Control: 2.189 OD450nm

Higher than 1.000 - Accepted

Cut-Off = 0.020 + 0.350 = 0.370

Calibrator: 0.556 - 0.559 - 0.564 OD450nm

Mean value: 0.540 OD450nm S/Co = 1.4

S/Co higher than 1.1 - Accepted

Sample 1: 0.070 OD450nm

Sample 2: 1.690 OD450nm

Sample 1 S/Co < 0.9 = negative

Sample 2 S/Co > 1.1 = positive

R. PERFORMANCES

Evaluation of Performances has been conducted in accordance to what reported in the Common Technical Specifications or CTS (art. 5, Chapter 3 of IVD Directive 98/79/EC).

1. LIMIT OF DETECTION

The limit of detection of the assay has been calculated by means of the British Working Standard for anti-HCV, NIBSC code: 99/538-003-WI. The table below reports the mean OD450nm values of this standard when diluted in negative plasma and then examined.

Dilution Factor	Lot #1 S/Co	Lot #2 S/Co
1 X	2.0	2.0
2 X	1.1	1.2
4 X	0.7	0.8
8 X	0.5	0.5
Negative plasma	0.3	0.3

In addition the sample coded Accurn 1 - series 3000 - supplied by Boston Biomedica Inc., USA, has been evaluated 'in toto' showing the results below.

CVAB/CE Lot ID	Series	S/Co
1201	3000	1.5
0502	3000	1.5
1202	3000	1.9

In addition, n° 7 samples, tested positive for HCV Ab with Ortho HCV 3.0 S/AVE, code 930820, lot. # EXE065-1, were diluted in HCV Ab negative plasma in order to generate limiting dilutions and then tested again on CVAB/CE, lot. # 1202, and Ortho. The following table reports the data obtained.

Sample n°	Limit Dilution	CVAB/CE S/Co	Ortho 3.0 S/Co
1	256 X	1.9	1.3
2	256 X	1.9	0.7
3	256 X	2.4	1.0
4	128 X	2.5	3.2
5	65 X	3.3	1.4
6	128 X	2.2	0.8
7	135 X	3.2	2.2

2. DIAGNOSTIC SPECIFICITY AND SENSITIVITY

The Performance Evaluation of the device was carried out in a trial conducted on more than total 5000 samples.

2.1 Diagnostic specificity:

It is defined as the probability of the assay of scoring negative in the absence of specific analyte. In addition to the first study, where a total of 5043 unselected blood donors (including 1st time donors), 210 hospitalized patients and 162 potentially interfering specimens (other infectious diseases, E.coli antibody positive, patients affected by non viral hepatic diseases, dialysis patients, pregnant women, hemodialyzed, lipemic, etc.) were examined, the diagnostic specificity was recently assessed by testing a total of 2876 negative blood donors on six different lots. A value of specificity of 100% was found.

No false reactivity due to the method of specimen preparation has been observed. Both plasma, derived with different standard techniques of preparation (citrate, EDTA and heparin), and sera have been used to determine the value of specificity. Frozen specimens have been tested, as well, to check for interferences due to collection and storage.
No interference was observed.

2.2 Diagnostic Sensitivity

It is defined as the probability of the assay of scoring positive in the presence of specific analyte.

The diagnostic sensitivity has been assessed externally on a total number of 359 specimens; a diagnostic sensitivity of 100% was found. Internally more than other 50 positive samples were tested, providing a value of diagnostic sensitivity of again 100%. Positive samples from infections carried out by different genotypes of HCV were tested as well.

Furthermore, most of seroconversion panels available from Boston Biomedica Inc., USA, (PHV) and Zeptematrix, USA, (HCV) have been studied.
Results are reported below for some of them.

Panel	N° samples	DiaPro®	Ortho***
PHV 901	11	9	9
PHV 904	7	2	4
PHV 905	9	3	4
PHV 906	7	7	7
PHV 907	7	3	2
PHV 908	13	10	8
PHV 909	3	2	2
PHV 910	5	3	3
PHV 911	5	3	3
PHV 912	3	1	1
PHV 913	4	2	2
PHV 914	9	5	5
PHV 915	4	3	0
PHV 916	8	4	3
PHV 917	10	6	6
PHV 918	8	2	0
PHV 919	7	3	3
PHV 920	10	6	6
HCV 10039	5	2	0
HCV 6212	9	6	7
HCV 10165	9	5	4

Note: * Positive samples detected

** HCV v.3.0

Finally the Product has been tested on the panel EFS Ac HCV, lot n° 0108.03.22C/01/A, supplied by the Etablissement Français Du Sang (EFS), France, with the following results:

EFS Panel Aa HCV

Sample	Lot # 1	Lot # 2	Lot # 2	Results
S/Co	S/Co	S/Co	S/Co	expected
HCV 1	2.2	2.4	2.6	positive
HCV 2	1.6	2.0	2.1	positive
HCV 3	1.5	1.7	1.6	positive
HCV 4	5.2	6.5	5.5	positive
HCV 5	1.6	1.8	1.6	positive
HCV 6	0.4	0.4	0.4	negative

3. **PRECISION:**
It has been calculated on two samples, one negative and one low positive, examined in 16 replicates in three separate runs. Results are reported as follows:

Lot # 1202

Negative Sample (N = 16)				
Mean values	1st run	2nd run	3 rd run	Average value
OD 450nm	0.094	0.099	0.096	0.096
Std Deviation	0.008	0.007	0.008	0.007
CV %	8.7	6.6	7.9	7.7

Cal # 2 - 7K (N = 16)

Mean values	1st run	2nd run	3 rd run	Average value
OD 450nm	0.396	0.403	0.418	0.406
Std Deviation	0.023	0.029	0.027	0.026
CV %	5.9	7.1	6.4	6.5
S/Co	1.1	1.1	1.2	1.1

Lot # 0602

Negative Sample (N = 16)				
Mean values	1st run	2nd run	3 rd run	Average value
OD 450nm	0.097	0.096	0.094	0.096
Std Deviation	0.009	0.010	0.008	0.009
CV %	8.9	10.1	8.4	9.1

Cal # 2 - 7K (N = 16)

Mean values	1st run	2nd run	3 rd run	Average value
OD 450nm	0.400	0.395	0.393	0.396
Std Deviation	0.021	0.025	0.026	0.024
CV %	5.4	6.2	6.6	6.1
S/Co	1.2	1.2	1.1	1.2

Lot # 0602/2

Negative Sample (N = 16)				
Mean values	1st run	2nd run	3 rd run	Average value
OD 450nm	0.087	0.091	0.088	0.089
Std Deviation	0.009	0.007	0.008	0.008
CV %	10.0	8.2	8.6	8.9

Cal # 2 - 7K (N = 16)

Mean values	1st run	2nd run	3 rd run	Average value
OD 450nm	0.386	0.390	0.391	0.389
Std Deviation	0.023	0.021	0.023	0.022
CV %	6.0	5.3	5.8	5.7
S/Co	1.1	1.2	1.2	1.2

The variability shown in the tables above did not result in sample misclassification.

S. LIMITATIONS

Repeatable false positive results, not confirmed by RIBA or similar confirmation techniques, were assessed as less than 0.1% of the normal population.
Frozen samples containing fibrin particles or aggregates after thawing have been observed to generate some false results.

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All the IVD Products manufactured by the company are under the control of a certified Quality Management System approved by an EC Notified Body. Each lot is submitted to a quality control and released into the market only if conforming with the EC technical specifications and acceptance criteria.

Manufacturer:

Dia.Pro. Diagnostico Bioprobes Srl.

Via G. Carducci n° 27 – Sesto San Giovanni (MI) – Italy



0318



Monobind Inc.

The World Resource for Diagnostic Products

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Orange County, California, January 10, 2020

IM Global Biomarketing Group - Moldova SRL,
Tighina str.65,office 607
MD-2001,Chisinau, Republic of Moldova

Commercialization Agreement

To Whom It May Concern:

We, Monobind Inc., an ISO 13485 certified company specializing in the research, development and manufacturing of in vitro diagnostic products for clinical and research application, located at 100 North Pointe Drive, Lake Forest, California 92630 USA;

Hereby authorizes and entitles IM Global Biomarketing Group from Moldova legally registered at Tighina str.65,office 607 MD-2001,Chisinau to effect clinical trials and evaluation of goods, registration of the goods at Health Ministry of Moldova, receive certificate of registration and conclude an agreement on consulting and examination of the documents needed for the registration in Moldova.

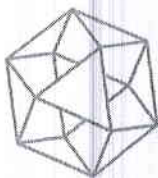
This is also to confirm that IM Global Biomarketing Group is the exclusive distributor our AccuBind® ELISA and AccuLite® CLIA products and accessories in Moldova. IM Global Biomarketing Group is authorized to promote and supply our products, to contract for their delivery and take part in tenders with our products.

This authorization is valid until January 1, 2021.

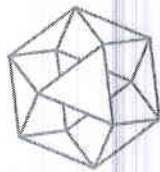
On behalf of the Monobind Inc.

Alicia Jerome Volkov
Marketing Director
Monobind Inc.





NSAI



NSAI

Annex to Certificate Number: MD19.4585

**Certificate of Registration
of Quality Management System**

to I.S. EN ISO 13485:2012

The National Standards Authority of Ireland certifies that:

Monobind Inc.

100 North Pointe Drive

Lake Forest, CA 92630

USA

has been assessed and deemed to comply with the requirements of the above standard in respect of the scope of operations given below:

The Design, Manufacture and Distribution of In-Vitro Diagnostic Medical Device Immunoassays, and Related Reagent Products and Equipment

Scope of Registration:

The Design, Manufacture and Distribution of In-Vitro Diagnostic Medical Device Immunoassays, and Related Reagent Products and Equipment

Activity	Location
Headquarters, Design, Manufacture	Monobind Inc. 100 North Pointe Drive Lake Forest, CA 92630 USA File No.: MD19.4585
Manufacture, Design	Monobind Inc. 103 North Pointe Drive Lake Forest, CA 92630 USA File No.: MD19.4585/A

Additional sites covered under this multi-site certification are listed on the Annex (File No. MD19.4585)

**Verified by:
Operations Manager**

Approved by:
Geraldine Larkin
Chief Executive Officer

Approved by:
Susan Murphy
European Medical Device
Operations Manager

Registration Number: MD19.4585
Certification Granted: May 18, 2010
Effective Date: Oct 29, 2017
Expiry Date: Oct 28, 2020



National Standards Authority of Ireland, 1 Swift Square, Northwood, Santry, Dublin 9, Ireland T +353 1 807 3800



DECLARATION OF CONFORMITY

Product Family TOTAL AND FREE PROSTATE SPECIFIC ANTIGEN (PSA and FPSA)

Specific Product Details						
Product Description	Item # ELISA	Item # CLIA	EDMS Code	GMDN ELISA Code	GMDN CLIA Code	Risk Class
Total PSA Immunoassay	2125-300	2175-300	12.03.01.32.00	54664	54665	High/ List B
Free PSA Immunoassay	2325-300	2375-300	12.03.01.33.00	54668	54669	High/ List B
Cancer VAST Immunoassay	8425-300	8475-300	12.03.01.32.00	54664	54665	High/ List B
Multi Ligand Control	ML-300	ML-300	12.03.01.32.00	38207	38207	High/ List B

Manufacturer

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Notified Body

Name NSAI
Body ID Number 0050
CE Cert # 304.1006
Registration # NL-CA002-2011-23306

Means of Conformity

Monobind Inc. declares that the product listed is in conformity with the Annex IV, IVD Type List B essential requirements and provisions of Council Directive: 98/79/EC

And is in conformance with the following standards:

EN 13612—2002 EN 980-2008 ISO 14971:2009
ISO 18113:2009 EN 13641:2002 EN 13640:2002
Under the principles of ISO 13485:2003

Signature

Place and date

Monobind Inc.

October 28, 2011

Signature

A Shatola

Name

Tony Shatola

Title QA Director

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AccuBind
ELISA Microwells

Total Prostate Specific Antigen (tPSA) Test System Product Code: 2125-300

1.0 INTRODUCTION

Intended Use: The Quantitative Determination of Total Prostate Specific Antigen (tPSA) Concentration in Human Serum by a Microplate Immunoassay.

2.0 SUMMARY AND EXPLANATION OF THE TEST

Prostate Specific Antigen (PSA) is a serine protease with chymotrypsin-like activity (1,2). The protein is a single chain glycoprotein with a molecular weight of 28.4 kDa (3). PSA derives its name from the observation that it is a normal antigen of the prostate but is not found in any other normal or malignant tissue.

PSA is found in benign, malignant and metastatic prostate cancer. Since prostate cancer is the second most prevalent form of male malignancy, the detection of elevated PSA levels plays an important role in the early diagnosis. Serum PSA levels have been found to be more useful than prostatic acid phosphatase (PAP) in the diagnosis and management of patients due to increased sensitivity (4).

In this method, PSA calibrator, patient specimen or control is first added to a streptavidin coated well. Biotinylated monoclonal and enzyme labeled antibodies (directed against distinct and different epitopes of PSA) are added and the reactants mixed. Reaction between the various PSA antibodies and native PSA forms a sandwich complex that binds with the streptavidin coated to the well.

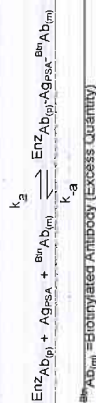
After the completion of the required incubation period, the enzyme-PSA antibody bound conjugate is separated from the unbound enzyme-PSA conjugate by aspiration or decantation. The activity of the enzyme present on the surface of the well is quantitated by reaction with a suitable substrate to produce color.

The employment of several serum references of known prostate specific antigen (PSA) levels permits the construction of a dose response curve of activity and concentration. From comparison to the dose response curve, an unknown specimen's activity can be correlated with PSA concentration.

3.0 PRINCIPLE

Immunoassay (TYPE 3):
The essential reagents required for an immunoassay are: enzyme, antibody, antigen, and substrate. In this procedure, the antigen (PSA) and the enzyme (PSA) are immobilized on the surface of a microplate well through the interaction of streptavidin coated on

the well and exogenously added biotinylated monoclonal anti-PSA antibody.
Upon mixing monoclonal biotinylated antibody, the enzyme-labeled antibody and a serum containing the native antigen, reaction results between the native antigen and the antibodies, without competition or steric hindrance, to form a soluble sandwich complex. The interaction is illustrated by the following equation:



Simultaneously, the complex is deposited to the well through the high affinity reaction of streptavidin and biotinylated antibody. This interaction is illustrated below:
$$\text{Enz-Ab}_{(p)}\text{-Ag-PSA-Ab}_{(m)} + \text{Streptavidin-coated well} \rightleftharpoons \text{Enz-Ab}_{(p)}\text{-Ag-PSA-Ab}_{(m)}\text{-Streptavidin-coated well}$$

Immobilized complex = complex bound to the solid surface
After equilibrium is attained, the antibody-bound fraction is separated from unbound antigen by decantation or aspiration. The enzyme activity in the antibody-bound fraction is directly proportional to the native antigen concentration. By utilizing several different serum references of known antigen values, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

4.0 REAGENTS

Materials Provided:

A. Prostate Specific Antigen (PSA) tPSA - Ions A-F
Six (6) vials of references PSA Antigen at levels of 0(A), 5(B), 10(C), 25(D), 50(E) and 100(F) ng/ml. Store at 2-8°C. A preservative has been added.

Note: The calibrators, human serum based, were calibrated using a reference preparation, which was assayed against the 1st IS 96/670.

B. PSA Enzyme Reagent - 13ml/vial - Icon[®]
One (1) vial containing enzyme labeled antibody, biotinylated monoclonal mouse IgG in buffer, dye, and preservative. Store at 2-8°C.

C. Streptavidin-Coated Plate - 96 wells - Icon[®]
One 96-well microplate coated with streptavidin and packaged in an aluminum bag with a drying agent. Store at 2-8°C.

D. Wash Solution Concentrate - 20 ml - Icon[®]
One (1) vial containing a surfactant in buffered saline. A preservative has been added. Store at 2-30°C.

E. Substrate A - 7ml/vial - Icon[®]
One (1) bottle containing tetramethylbenzidine (TMB) in buffer. Store at 2-8°C.

F. Substrate B - 7ml/vial - Icon[®]
One (1) bottle containing hydrogen peroxide (H₂O₂) in buffer. Store at 2-8°C.

G. Stop Solution - 8ml/vial - Icon[®]
One (1) bottle containing a strong acid (1N HCl). Store at 2-30°C.

H. Product Instructions.
Note 1: Do not use reagents beyond the kit expiration date.

Note 2: Do not use reagents that are contaminated or have bacteria growth.
reagents are stable for sixty (60) days when stored at 2-8°C. Kit and component stability are identified on the label.

Note 3: Above reagents are for a single 96-well microplate

4.1 Required But Not Provided:

1. Pipette(s) capable of delivering 25 & 50µl volumes with a precision of better than 1.5%.
2. Dispenser(s) for repetitive deliveries of 0.100ml and 0.350ml volumes with a precision of better than 1.5%.
3. Microplate washers or a squeeze bottle (optional).
4. Microplate Reader with 450nm and 620nm wavelength absorbance capability.
5. Absorbent Paper for blotting the microplate wells.
6. Plastic wrap or microplate cover for incubation steps.
7. Vacuum aspirator (optional) for wash steps.
8. Timer.
9. Quality control materials

5.0 PRECAUTIONS

For In Vitro Diagnostic Use

Not for Internal or External Use in Humans or Animals

All products that contain human serum have been found to be non-reactive for Hepatitis B Surface Antigen, HIV 1&2 and HCV Antibodies by FDA licensed reagents. Since no known test can offer complete assurance that infectious agents are absent, all human serum products should be handled as potentially hazardous and capable of transmitting disease. Good laboratory procedures for handling blood products can be found in the Center for Disease Control / National Institute of Health, "Biosafety in Microbiological and Biomedical Laboratories," 2nd Edition, 1988, HHS Publication No. (CDC) 88-8335.

Safe Disposal of kit components must be according to local regulatory and statutory requirement.

6.0 SPECIMEN COLLECTION AND PREPARATION

The specimens shall be blood, serum in type and the usual precautions in the collection of venipuncture samples should be observed. For accurate comparison to established normal values, a fasting morning serum sample should be obtained. The blood should be collected in a plain red top venipuncture tube without additives or anti-coagulants. Allow the blood to clot. Centrifuge the specimen to separate the serum from the cells. Samples may be refrigerated at 2-8°C for a maximum period of five (5) days. If the specimen(s) cannot be assayed within this time, the sample(s) may be stored at temperatures of -20°C for up to 30 days. Avoid use of contaminated devices. Avoid repetitive freezing and thawing. When assayed in duplicate, 0.050ml of the specimen is required.

7.0 QUALITY CONTROL

Each laboratory should assay controls at levels in the low, normal and elevated range for monitoring assay performance. These controls should be treated as unknowns and values determined in every test procedure performed. Quality control charts should be maintained to follow the performance of the supplied reagents. Pertinent statistical methods should be employed to ascertain trends. Significant deviation from established performance can indicate unnoticed change in experimental conditions or degradation of kit reagents. Fresh reagents should be used to determine the reason for the variations.

8.0 REAGENT PREPARATION:

1. Wash Buffer
Dilute contents of wash concentrate to 1000ml with distilled or deionized water in a suitable storage container. Store at room temperature 20-27°C for up to 60 days.
 2. Working Substrate Solution
Pour the contents of the amber vial labeled Solution 'A' into the clear vial labeled Solution 'B'. Place the yellow cap on the 'clear-vial' for easy identification. Mix and label accordingly. Store at 2-8°C.
- Note 1: Do not use the working substrate if it looks blue.

Note 2: Do not use reagents that are contaminated or have bacteria growth.

9.0 TEST PROCEDURE

Before proceeding with the assay, bring all reagents, serum references and controls to room temperature (20 - 27°C).

Test Procedure should be performed by a skilled individual or trained professional

1. Format the microplates' wells for each serum reference, control and patient specimen to be assayed in duplicate. Replace any unused microwell strips back into the aluminum bag, seal and store at 2-8°C.

2. Pipette 0.025 ml (25µl) of the appropriate serum reference, control or specimen into the assigned well.

3. Add 0.100 ml (100µl) of the PSA Enzyme Reagent to each well. It is very important to dispense all reagents close to the bottom of the coated well.

4. Swirl the microplate gently for 20-30 seconds to mix and cover.

5. Incubate 30 minutes at room temperature.

6. Discard the contents of the microplate by decantation or aspiration. If decanting, tap and blot the plate dry with absorbent paper.

7. Add 350µl of wash buffer (see Reagent Preparation Section), decant (tap and blot) or aspirate. Repeat two (2) additional times for a total of three (3) washes. An automatic or manual plate washer can be used. Follow the manufacturer's instruction for proper usage. If a squeeze bottle is employed, fill each well by dispensing the container (avoiding air bubbles) to dispense the wash. Decant the wash and repeat two (2) additional times.

8. Add 0.100 ml (100µl) of working substrate solution to all wells (see Reagent Preparation Section). Always add reagents in the same order to minimize reaction time differences between wells.

DO NOT SHAKE THE PLATE AFTER SUBSTRATE ADDITION

9. Incubate at room temperature for fifteen (15) minutes.

10. Add 0.050ml (50µl) of stop solution to each well and mix gently for 15-20 seconds. Always add reagents in the same order to minimize reaction time differences between wells.

11. Read the absorbance in each well at 450nm (using a reference wavelength of 620-630nm to minimize well imperfections) in a microplate reader. The results should be read within thirty (30) minutes of adding the stop solution.

10.0 CALCULATION OF RESULTS

A dose response curve is used to ascertain the concentration of PSA in unknown specimens.

1. Record the absorbance obtained from the printout of the microplate reader as outlined in Example 1.
2. Plot the absorbance for each duplicate serum reference versus the corresponding PSA concentration (in ng/ml) on linear graph paper (do not average the duplicates of the serum references before plotting).
3. Draw the best-fit curve through the plotted points.
4. To determine the concentration of PSA for an unknown, locate the average absorbance of the duplicates for each unknown on the vertical axis of the graph, find the intersecting point on the curve, and read the concentration (in ng/ml) from the horizontal axis of the graph (the duplicates of the unknown may be averaged as indicated). In the following example, the average absorbance (1.142) intersects the dose response curve at (23.6 ng/ml) PSA concentration (See Figure 1).

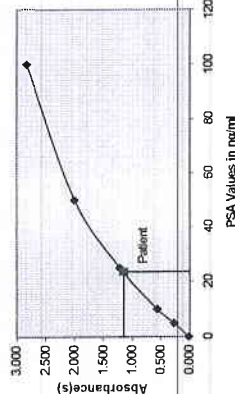
Note: Computer data reduction software designed for ELISA assays may also be used for the data reduction. If such software is utilized, the validation of the software should be ascertained.

EXAMPLE 1

Sample I.D.	Well Number	Abs (A)	Mean Abs (B)	Value (ng/ml)
Cal A	A1	0.019	0.019	0
	B1	0.019		
Cal B	C1	0.279	0.276	5
	D1	0.273		
Cal C	E1	0.567	0.563	10
	F1	0.559		
Cal D	G1	1.248	1.213	25
	H1	1.179		
Cal E	A2	2.051	1.999	50
	B2	1.947		
Cal F	C2	2.892	2.833	100
	D2	2.775		
Patient	E3	1.186	1.142	23.6
	F3	1.099		

*The data presented in Example 1 and Figure 1 is for illustration only and should not be used in lieu of a dose response curve prepared with each assay.

Figure 1



11.0 Q.C. PARAMETERS

In order for the assay results to be considered valid the following criteria should be met:

1. The absorbance (OD) of calibrator F should be ≥ 1.3 .
2. Four out of six quality control pools should be within the established ranges.

12.0 RISK ANALYSIS

The MSDS and Risk Analysis Form for this product is available on request from Monobind Inc.

- 12.1 Assay Performance
 1. It is important that the time of reaction in each well is held constant to achieve reproducible results.
 2. Pipetting of samples should not extend beyond ten (10) minutes to avoid assay drift.

3. Highly lipemic, hemolyzed or grossly contaminated specimen(s) should not be used.
4. If more than one (1) plate is used, it is recommended to repeat the dose response curve.
5. The addition of substrate solution initiates a kinetic reaction. Therefore, the substrate and stop solution should be added in the same sequence to eliminate any time deviation during reaction.
6. Plate readers measure vertically. Do not touch the bottom of the wells.
7. Failure to remove adhering solution adequately in the application or decantation wash step(s) may result in poor replication and spurious results.
8. Use components from the same lot. No intermixing of reagents from different batches.
9. Patient specimens with PSA concentrations above 100 ng/ml may be diluted (for example 1/10 or higher) with normal female serum (PSA = 0 ng/ml) and re-assayed. The sample's concentration is obtained by multiplying the result by the dilution factor (10).
10. Accurate and precise pipetting, as well as following the exact time and temperature requirements prescribed are essential. Any deviation from Monobind's IFU may yield inaccurate results.
11. All applicable national standards, regulations and laws, including, but not limited to, good laboratory procedures, must be strictly followed to ensure compliance and proper device usage.
12. It is important to calibrate all the equipment e.g. Pipettes, Readers, Washers and/or the automated instruments used with this device, and to perform routine preventative maintenance.
13. Risk Analysis- as required by CE Mark IVD Directive ISO 14971:2009 - for this and other devices, made by Monobind, can be requested via email from Monobind@monobind.com.

12.2 Interpretation

1. **Measurements and Interpretation of results must be performed by a skilled individual or trained professional.**
2. Laboratory results alone are only one aspect for determining patient care and should not be the sole basis for therapy, particularly if the results conflict with other determinants.
3. For valid test results, adequate controls and other parameters must be within the listed ranges and assay requirements.
4. If test kits are altered, such as by mixing parts of different kits, which could produce false test results, or if results are incorrectly interpreted, Monobind shall have no liability.
5. If computer controlled data reduction is used to interpret the results of the test, it is imperative that the predicted values for the calibrators fall within 10% of the assigned concentrations.
6. PSA is elevated in benign prostatic hypertrophy (BPH). Clinically an elevated PSA value alone is not of diagnostic value as a specific test for cancer and should only be used in conjunction with other clinical manifestations (observations) and diagnostic procedures (prostate biopsy). Free PSA determinations may be helpful in regard to the discrimination of BPH and prostate cancer conditions (5).

13.0 EXPECTED RANGES OF VALUES

Healthy males are expected to have values below 4 ng/ml (4).

TABLE 1
Expected Values for the PSA Elisa Test System

Healthy Males	<4 ng/ml
---------------	----------

It is important to keep in mind that establishment of a range of values which can be expected to be found by a given method for a population of normal persons is dependent upon a multiplicity of factors: the specificity of the method, the population tested and the precision of the method in the hands of the analyst. For these reasons, each laboratory should depend upon the range of expected values established by the Manufacturer only until an in-house range can be determined by

the analysts using the method with a population indigenous to the area in which the laboratory is located.

14.0 PERFORMANCE CHARACTERISTICS

14.1 Precision
The within and between assay precisions of the IPSA AccuBind™ ELISA test system were determined by analyses on three different levels of control sera. The number, mean value, standard deviation and coefficient of variation for each of these control sera are presented in Table 2 and Table 3.

TABLE 2
Within Assay Precision (Values in ng/ml)

Sample	N	X	S.D.	C.V.
Level 1	20	0.7	0.05	7.1%
Level 2	20	4.5	0.20	4.4%
Level 3	20	28.3	1.07	3.7%

TABLE 3

Between Assay Precision* (Values in ng/ml)

Sample	N	X	S.D.	C.V.
Level 1	10	0.8	0.09	11.3%
Level 2	10	4.3	0.25	5.8%
Level 3	10	27.5	1.42	5.2%

*As measured in ten experiments in duplicate.

14.2 Sensitivity

The IPSA AccuBind™ ELISA test system has a sensitivity of 0.012 ng. This is equivalent to a sample containing 0.5 ng/ml IPSA concentration.

14.3 Accuracy

The IPSA AccuBind™ ELISA method was compared with a reference ELISA method. Biological specimens from low, normal, and elevated concentrations were assayed. The total number of such specimens was 241. The least square regression equation and the correlation coefficient were computed for the IPSA AccuBind™ ELISA test method in comparison with the reference method. The data obtained is displayed in Table 4.

TABLE 4
Least Square

Method	Mean	Regression Analysis	Correlation Coefficient
This Method (X)	5.62	$y = -0.0598 + 0.98(X)$	0.987
Reference (Y)	5.57		

Only slight amounts of bias between the IPSA AccuBind™ ELISA method and the reference method are indicated by the closeness of the mean values. The least square regression equation and correlation coefficient indicates excellent method agreement.

14.4 Specificity:

No interference was detected with the performance of IPSA AccuBind™ ELISA test system upon addition of massive amounts of the following substances to a human serum pool.

Acetylsalicylic Acid	100 µg/ml
Ascorbic Acid	100 µg/ml
Caffeine	10 µg/ml
CEA	10 µg/ml
AFP	10 µg/ml
CA-125	1000 U/ml
HCG	1000 U/ml
hLH	10 U/ml
hTSH	100 mIU/ml
hPRL	100 µg/ml

15.0 REFERENCES

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Revision: 3 Date: 08/15/11 DCO: 0524

Cat #: 2125-300

Size	30(A)	30(B)
A)	1ml set	1ml set
B)	1 (13ml)	2 (13ml)
C)	1 plate	2 plates
D)	1 (20ml)	1 (20ml)
E)	1 (7ml)	2 (7ml)
F)	1 (7ml)	2 (7ml)
G)	1 (6ml)	2 (6ml)

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