HBsAg

Confirmation

Set of Reagents for the confirmation of HBsAg positivity in human sera or plasma

- for “in vitro” diagnostic use only -

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Code SCONF.CE
20/40 tests

HBsAg Confirmation

A. INTENDED USE

In the screening of blood units for Hepatitis B surface Antigen or HBsAg some false positivity may happen, leading to a misinterpretation of the assay results and a misclassification of the blood unit and the donor.
To confirm the positivity of a screened sample or to confirm the presence of an ongoing HBV infection in a hospitalized patient, a confirmatory test has to be run.
A simple procedure based on an immunoreaction of neutralization is used in combination with the HBsAg assay.

B. PRINCIPLE OF THE ASSAY

The device has to be used in combination with the products code SAG1.CE/SAG1ULTRA.CE for the determination of HBsAg in human sera and plasma.
The sample, whose repeatedly positivity for HBsAg has to be confirmed, is premixed with a reagent containing high titer anti HBsAg antibodies that will neutralize the antigen is really present in the sample.
The neutralized sample is then tested for HBsAg according to the procedure reported for the specific device.
If the positivity in the first screening test is specifically related to the presence of HBsAg in the sample, the same will not react any more in the assay having been neutralized by the antibody.
If at contrary the positivity of the sample is not abolished by the neutralization reaction, this reactivity is not due specifically to the presence of HBsAg in the sample, but to some interfering substance.

C. CONTENT OF THE KIT

The set contains the following reagents**.**

1. Neutralizing Reagent SOLN NEUT
It contains high titer human plasma positive for anti HBsAg antibodies, 0.2 mg/ml gentamicine sulphate and 0.1% Kathon GC as preservatives.

2. Control Reagent CONTROL
It contains human plasma negative for anti HBsAg antibodies, 0.2 mg/ml gentamicine sulphate and 0.1% Kathon GC as preservatives.

3. Assay Diluent DILSPE
0.15 M NaCl phosphate buffered solution pH 7.0 ± 0.2 containing 0.1% Kathon GC for the dilution of over ranging samples .

Note: *Reagents have been tested and found negative for HBsAg, HCV Ab and HIV Ab with CE-marked kits.*

Number of tests	**20**	**40**
Code	**SCONF.CE**	**SCONF.CE.40**
Control Reagent (CONTROL)	1x4ml/vial	1x8ml/vial
Neutralizing Reagent (SOLN NEUT)	1x4ml/vial	1x8ml/vial
Phosphate Buffered Saline (DILSPE)	1x30ml/vial	1x60ml/vial
Package insert	N°1	N°1

D. MATERIALS REQUIRED BUT NOT PROVIDED

1. CE marked devices for HBsAg determination:

Product	**Code**	**Tests**
HBsAg one	SAG1.CE	192
	SAG1.CE.96	96
	SAG1.CE.480	480
	SAG1.CE.960	960
HBsAg one version ULTRA	SAG1ULTRA.CE	192
	SAG1ULTRA.CE.96	96
	SAG1ULTRA.CE.480	480
	SAG1ULTRA.CE.960	960

2. Isotonic sterile solution.
3. Calibrated Micropipettes and disposable plastic tips.
4. Timer with 60 minute range or higher.
5. Absorbent paper tissues.
6. Calibrated ELISA microplate thermostatic incubator (dry or wet), capable to provide shaking at 1300 rpm+/-150, set at +37°C.
7. Calibrated ELISA microwell reader with 450nm (reading) and with 620-630nm (blanking) filters.
8. Calibrated ELISA microplate washer.
9. Vortex or similar mixing tools.
10. Disposable plastic tube of 2-5 ml.

E. WARNINGS AND PRECAUTIONS

1. For “in vitro” diagnostic use only.
2. The Set has to be used by skilled and properly trained technical personnel only, under the supervision of a medical doctor responsible of the laboratory.
3. When the device is used for confirmation of a sample repeatedly positive in 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.
4. The laboratory environment should be controlled so as to avoid contaminants such as dust or air-born microbial agents, when opening the vials contained in the set.
5. Upon receipt, store the kit at 2..8°C into a tem perature controlled refrigerator or cold room.
6. Do not interchange Reagents between different lots of the device. It is even recommended that Reagents between two sets of the same lot are not interchanged.
7. Check that the Reagents of the device 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.
8. Avoid cross-contamination between kit reagents by using disposable tips and changing them between the use of each one.
9. Do not use the set after the expiration date stated on the external container and internal (vials) labels.
10. 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.
11. Waste produced during the use of the set in combination with the devise for HBsAg determination 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..
12. 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.
13. 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.
14. Refer to the Instructions for Use of the product code SAG1.CE/SAG1ULTRA.CE used in combination for the confirmation assay.

F. SPECIMEN: PREPARATION AND WARNINGS

1. The sample turned out to be repeatedly positive in the first HBsAg determination with HBsAg One has to be used for the test of neutralization. Treat the sample as described in section L.
2. Avoid any addition of preservatives to samples after first screening; 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 (red) and lipemic ("milky") samples have to be discarded by definition anyway as they could generate false results in the test for HBsAg.
5. Samples containing residues of fibrin or heavy particles or microbial filaments and bodies should be discarded as well as they could give rise to false positive results both in HBsAg first assay and even in the confirmation one.
6. The assay is not suitable to confirm the negativity of samples that turned out to be negative in the first HBsAg screening test.
7. Sera and plasma can be stored at +2°..8°C for up t o five days after collection. For longer storage periods, samples can be stored frozen at –20°C for several months.
8. Any frozen sample should not be frozen/thawed more than once as this may generate particles that could affect the test result. If some turbidity is present or presence of micro particles is suspected after thawing, filter the sample on a disposable 0.2-0.8u filter to clean it up for testing or use the two-steps alternative method.
9. Refer to the Instructions for Use of the products code SAG1.CE/SAG1ULTRA.CE used in combination for the confirmation assay.

G. 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 $\pm$2%.
2. The **ELISA incubator** has to be set at +37°C (tolerance of $\pm$1°C) and regularly checked to ensure the correc t 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 $\pm$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 performances 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 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 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 $\leq$ 10 nm; (b) absorbance range from 0 to $\geq$ 2.0; (c) linearity to $\geq$ 2.0; 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. **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.

H. PRE ASSAY CONTROLS AND OPERATIONS

1. Check the expiration date of the set printed on the external label of the kit box. Do not use if expired.
2. Check that the liquid components of the set are not contaminated by naked-eye visible particles or aggregates.
3. Allow components to reach room temperature (about 1 hr) and then mix them on vortex.
4. Check that micropipettes are set to the required volume.
5. Check that equipments and the kits SAG1.CE/SAG1ULTRA.CE used in combination are available and ready to use.
6. In case of problems, do not proceed further with the test and advise the supervisor.
7. Refer to the Instructions for Use of the product code SAG1.CE/SAG1ULTRA.CE used in combination for the confirmation assay.

I. ASSAY PROCEDURE

The confirmation assay reported below has to be carried out on a sample repeatedly positive for HBsAg, when the product code SAG1.CE/SAG1ULTRA.CE is used for first screening.
The test is not suitable to confirm negative samples.
The Negative Control and the Calibrator of the kit code SAG1.CE/SAG1ULTRA.CE have always to be run whenever the assay of confirmation is used.

Samples with OD450nm < 2

If the sample gave an optical signal < 2 OD450nm in the screening test use the following distribution protocol:

1. Add 50 ul Neutralizing Reagent to 150 ul sample to be confirmed in a disposable test tube (**N**). Mix on vortex.
2. Add 50 ul Control Reagent to 150 ul sample to be confirmed in a second disposable test tube (**C**). Mix on vortex.
3. Incubate both tubes for 30 min at room temperature.
4. Then follow the Instructions for Use of the product code SAG1.CE/SAG1ULTRA.CE and determine HBsAg reactivity in both N and C.

Samples with OD450nm > 2

If the sample gave an optical signal > 2.000 OD450nm in the screening test, use the following procedure:

1. Dilute the sample 1:100 by dispensing 5 ul of specimen and 495 ul of Assay Diluent in a disposable tube (S01K). Mix on vortex.
2. Dilute further 1:10,000 the sample by dispensing 5 ul of the 1:100 solution and 495 ul of Assay Diluent in a disposable tube (S10K). Mix on vortex.
3. In a first test tube dispense 150 ul S01K and add 50 ul control Reagent (**C01K**). Mix on vortex.
4. In a second test tube dispense again 150 ul S01K and add 50 ul Neutralizing Reagent (**N01K**). Mix on vortex.
5. In a third test tube dispense 150 ul of solution S10K and add 50 ul Control Reagent (**C10K**). Mix on vortex.
6. In a fourth test tube dispense again 150 ul of solution S01K and add 50 ul Neutralizing Reagent (**N10K**). Mix on vortex.
7. Incubate all these tubes for 30 min at room temperature.
8. Then follow the Instructions for Use of the product code SAG1.CE/SAG1ULTRA.CE and determine HBsAg reactivity in all the tubes (C01K, C10K, N01K and N10K).
9. If the OD450nm value of the 1:10,000 dilution is still greater than 2 for the non-neutralized sample (control well), repeat the test after further diluting the sample 1:100,000.

An example of dispensation scheme is reported in the following table:

Microplate

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

Legenda : BLK = Blank NC = Negative Control CAL = Calibrator S = Sample C = Control N = Neutralizing Reagent

L. CALCULATION OF RESULTS

The positivity of the specimen is confirmed if the ratio between the OD450nm value for the control well (**C**) and the OD450nm value for the neutralization well (**N**) is higher than 2, that is formulated mathematically as follows:

$$C / N > 2$$

If a HBsAg positive sample shows a ratio C/N < 2 in the neutralization assay it is considered false positive.

M. INTERNAL QUALITY CONTROL

A check is performed any time the kit is used in combination with the device for HBsAg determination (code SAG1.CE/SAG1ULTRA.CE) in order to assure full matching the expected performances.

In particular ensure that the following results are met:

Parameter	Requirements
Blank well	< 0.100 OD450nm value
Negative Control (NC) of product code SAG1.CE/SAG1ULTRA.CE	< 0.050 mean OD450nm value after blanking
Calibrator (CAL) of product code SAG1.CE/SAG1ULTRA.CE treated with CONTROL	S/Co $\geq$ 2
Calibrator (CAL) of product code SAG1.CE/SAG1ULTRA.CE treated with SOLN NEUT	C/N > 2
Sample to be confirmed treated with CONTROL	S/Co > 1.1

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	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.
Calibrator (CAL) Treated with CONTROL S/Co < 2	1. that the procedure has been correctly performed; 2. that no mistake has occurred during the distribution of the Calibrator (dispensation of negative control instead) or the CONTROL (dispensation of the SOLN NEUT instead). 3. that the washing procedure and the washer settings are as validated in the pre qualification study; 4. that no external contamination has occurred.
Calibrator (CAL) treated with SOLN NEUT C/N < 2	1. that the procedure has been correctly performed; 2. that no mistake has occurred during the distribution of the Calibrator (dispensation of negative control instead) or the SOLN NEUT (dispensation of the CONTROL instead). 3. that the washing procedure and the washer settings are as validated in the pre qualification study; 4. that no external contamination has occurred.
Sample to be confirmed treated with CONTROL S/Co < 1.1	1. the sample to be confirmed was mishandled or confused with a negative one. 2. that the SOLN NEUT was dispensed instead of the CONTROL. 3. that the washing procedure and the washer settings are as validated in the pre qualification study.

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

N. EXAMPLE OF RESULTS

Below an example of calculation and interpretation of results is reported:

Sample # 1

Control well C: 1.000 OD450nm
Neutralization well N: 0.100 OD450nm
Ratio C/N: 10
Result of confirmation: **true positive**

Sample # 2

Control well C: 1.000 OD450nm
Neutralization well N: 0.800 OD450nm
Ratio C/N: 1.25
Result of confirmation: **false positive**

Important note:

*If the OD450nm value for the control well (**C**) of the 1:100,000 dilution of the sample is still higher than the upper limit of detection of the microplate reader, the specimen is confirmed positive if the value for the neutralization well (**N**) is equal or less than 50% of the maximum optical density of the reader. An example of such case is reported below:*

Upper limit of detection of the reader: 2.000

Sample # 1 diluted 1:100,000

Control well C: >2.000 OD450nm
Neutralization well N: 0.800 OD450nm
50% of *the upper limit of detection of the reader*:: 1.000
Result of confirmation: ***true positive***

Sample # 2 diluted 1:100,000

Control well C: >2.000 OD450nm
Neutralization well N: 1.850 OD450nm
50% of *the upper limit of detection of the reader*::1.000
Result of confirmation: ***false positive***

O. TEST PERFORMANCES

Sensitivity:

A total of 300 samples positive for HBsAg in HBsAg One, including standards for HBsAg provided by WHO, NIBSC and PEI, were examined. In the study also 15 panels of HBsAg seroconversion were included.
All the positive samples were confirmed positive providing a value of 100% sensitivity.

Specificity:

A total of 20 false positive samples (prevalently HAMA positive), obtained from a population examined without the HAMA blocker with the kits SAG1.CE/SAG1ULTRA.CE, were tested.
All of them, again tested with a SAG1.CE/SAG1ULTRA.CE device lacking the HAMA blocker, were not confirmed for HBsAg presence and therefore defined false positives.
In addition, even if the assay is not suitable to test negative samples, a total of 50 specimens negative in HBsAg One first screening, coming from hospitalized patients with pathologies different from HBV infection, showed a mean C/N value < 2 in the confirmation assay, therefore proving the validity of the above calculation and not to generate interferences in the confirmation test.

P. LIMITATIONS OF THE TEST

All the limitations reported in the kit HBsAg One apply to the above described assay, as they are conducible to the HBsAg assay itself.
Please read with attention the Instructions for Use of the product code SAG1.CE/SAG1ULTRA.CE before carrying out the test of neutralization.

In particular the product must not be applied to those specimens showing particles or aggregates, unless the sample is cleaned before use by filtration on 0.2-0.8 u disposable filters.

The confirmation assay for HBsAg positivity is not suitable to confirm negativity on negative samples and therefore must not be used for such analysis.

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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.

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