HeMoStep™ kit

Measurement of peripheral blood contamination in CSF

REF | Σ | IMS1510 | 100 test | CE IVD

1. INTENDED USE

The HeMoStep kit is an in vitro immunoassay for the quantitative measurement of blood contamination of cerebrospinal fluid (CSF) samples by flow cytometry.

The intended use of the assay is to quantify the contamination of CSF samples with peripheral blood, helping, in combination with other tests such as flow cytometry (FCM) and cytology (CC), to the accurate interpretation of the results from the analysis of this type of samples (CSF) improving the diagnosis of leptomeningeal disease in patients with B and T cell lymphomas and acute leukemias of lymphoid and myeloid origin.

2. APPLICATION FIELD

CSF fluid cell analysis is an important clinical procedure for the diagnosis, classification and prognosis of a wide variety of diseases. CSF sampling is performed by a procedure called lumbar puncture (LP), which involves inserting a needle into the spinal canal. During the procedure, the needle passes through several layers of vascularised tissue until it reaches the subarachnoid space and peripheral blood (PB) may be introduced into the sample tube contaminating the CSF. In addition, the puncture can sometimes be complicated by increased bleeding, called traumatic LP (up to 20%), resulting in visible red contamination of the CSF sample. This type of puncture is called traumatic[1]. Thus, the presence of blood cells and the affected concentration of some substances due to blood contamination in the CSF can complicate the analysis and confuse the diagnosis.

Multiparametric FCM immunophenotyping combines high specificity with good clinical sensitivity and several studies and guidelines recommend this immunophenotyping for efficient and reliable CSF diagnosis in patients with haematological malignancies such as B- and T-cell lymphomas and acute leukaemias of lymphoid and myeloid origin, in whom CSF tumour infiltration is suspected[2-4]. Assessment of CSF contamination with SP should be performed in all cases and especially in cases where the presence of blood cells is observed and when malignant cells are present in peripheral blood. In this sense, although the cerebrospinal fluid is crystalline, it is common for it to present an alteration in colour due either to pathological bleeding in the central nervous system (CNS) or, as we have already mentioned, to a traumatic puncture. In the latter case, it is difficult to evaluate the degree of contamination by visual inspection alone, as the visual threshold for the perception of blood in cerebrospinal fluid varies from 400 to 6000 red blood cells (RBCs) per mm^3, according to different authors[1,5-6] and even visibly undetectable blood contamination can drastically alter the CSF content.

Some of the most commonly used methods for estimation of CSF contamination by CMF are based on immunophenotyping and enumeration of absolute contaminating cells from peripheral blood, mainly RBCs and/or neutrophils. However, such protocols have limitations (low precision and low sensitivity) related to cell loss during the concentration/centrifugation steps, but also to cell destruction due to the rapid in vitro cytotoxic effects of CSF on leukocytes and thus on neutrophils[7].

The assessment of CSF sample quality is essential in the diagnosis and follow-up of leukaemias and lymphomas and having a method that circumvents the disadvantages associated with the cell destruction of this type of analysis can be a determining factor in obtaining good results.

This kit, based on the quantification of total haemoglobin (Hb), a specific biomarker for RBCs, helps to interpret diagnostic results, as it circumvents the problems arising from CSF cytotoxicity, minimizes sample usage, which is particularly important in paucicellular samples[2,8] and significantly improves sensitivity for the determination of CSF contamination with PB.

3. PRINCIPLE OF METHODOLOGY

The HeMoStep kit is a monoplex (single population) sandwich immunoassay that allows the capture of a soluble analyte (Hb).

The assay is based on beads coated with a capture antibody and internally fluorescently labelled at FL3 (685 nm), presenting a discrete fluorescence intensity pattern with very little transfer to FL1 (519 nm) or FL2 (578 nm), leaving other detectors available for analyte determination. In a first step, the sample is incubated with the beads, allowing the antibody coating the beads to capture the analyte present in the sample. The beads are then washed to remove the rest of the sample and subsequently incubated with the detector antibody, fluorescently conjugated with Phycoerythrin (PE). After a second wash and resuspension the beads can be analysed using a flow cytometer. The fluorescence intensity is proportional to the amount of analyte present in the sample.

The assay can be performed on any conventional flow cytometer and is for professional use.

4. REAGENTS

a) Kit contents

The reagents included in one kit are sufficient to perform 100 determinations. Each HeMoStep kit contains:

INCUB	**Incubation buffer for HeMoStep kit.** One vial with 5.5 ml (45 µl/test).
BEADS	**Capture microspheres coated with an Hb-specific monoclonal antibody.** Magnetic polystyrene microspheres (mean diameter 6 µm), are supplied in 1 vial at the following concentration: 2000 microspheres/test (5 µl/test) and in a buffered aqueous solution containing protein stabilizer and 0.09% sodium azide (NaN_3), as an anti-microbial agent.
WASHBUF 10X	**25 ml wash buffer (10X).** PBS 10% BSA, pH 7.4 - 10X. Contains 10% albumin in 10mM sodium phosphate, 150mM NaCl, pH 7.4, contains KATHON™ anti-microbial agent. Dilute the contents of the 10X assay buffer to 1X (PBS 1% BSA) in PBS, pH 7.4, for use in this assay.
CNTRL+	**Positive control. 5 vials of lyophilized RBCs lysate.** Solubility H_2O. Reconstitute before use.
CAL	**Calibration microspheres capped with two different concentrations of Hb.** 2 different populations of magnetic polystyrene microspheres (mean diameter 6 µm) and an internal fluorescence standard different from the capture microspheres are supplied in 1 vial at the following concentration: 2000 (1000 beads of each) microspheres/test (5 µl/test) - 100 µl/vial and in a buffered aqueous solution containing protein stabilizer and 0.09% sodium azide (NaN_3) as an anti-microbial agent.
STD	**Standard of known concentration. 1 vial of lyophilized RBCs lysate with known concentrations of [Hb].** Solubility H_2O. Reconstitute before use. To be used to generate the standard curve.
CONJ	**1.2 ml fluorescently conjugated (PE) detector antibody (10 µl/test).** The antibody is supplied in 2 vials (0.6 ml/vial), use concentration and buffered aqueous solution containing protein stabilizer and 0.09% sodium azide (NaN_3) as an antimicrobial agent.
	Instructions for use.

b) Materials, reagents and equipment required not supplied

- Flow cytometer equipped with at least one blue laser, 488 nm, and fluorescent channels for PE (Ex-Max 496 nm/Em-Max 578 nm) and PerCP (Ex-Max 482 nm/Em-Max 678 nm).
- Adjustable calibrated micropipettes covering a range of 1-1000 µL and corresponding disposable pipette tips.
- Pipette tips.
- Pasteur pipettes.
- Magnetic rack; MagneSphere(R) Mag. Sep. Stand 12- hole, 12x75mm (PROMEGA, Ref Z5343).
- 12x75 mm Polystyrene round-bottomed tubes (Cytometer tubes).
- 1,5 ml Eppendorf tube.
- FACS Lysing solution (BD Biosciences, Catalog No. 349202), to be used during the protocol as sample diluent DILUB and negative control CNTRL-.
- PBS; pH 7.4 (phosphate buffered saline) 1X, to be used for 10X wash buffer dilution and sample preparation.
- Disinonised or distilled H2O.
- Timer.
- Disposable gloves.
- Waste container for biological substances.

5. STORAGE AND HANDLING CONDITIONS

Store refrigerated between +2 and +8°C. DO NOT FREEZE.

The unopened kit is stable until the expiry date. Do not use after this date. After opening, reagents are stable if stored at +2 to +8°C and protected from contamination. With the exception of the standard and positive controls which, once reconstituted, should be used immediately. If it is not possible to use them immediately or there is a surplus after use, it is recommended to discard them or as a last resort to freeze them (-20°C) where they can remain stable for a few days (e.g. 5 days). Do not leave the reagents open and at a temperature different from the storage temperature for longer than strictly necessary.

6. RECOMMENDATIONS AND WARNINGS

- FOR IN VITRO DIAGNOSTIC USE. For professional use only.
- Only for qualified laboratory personnel.
- Kit components contain KATHON™ or sodium azide (NaN3). Compounds should be dissolved with tap water before disposal. These conditions are recommended to avoid deposits in pipes. Material Safety Data Sheet (MSDS) available on the website www.immunostep.com
- Before starting the analysis, read the instructions carefully. Deviations from the recommended procedures may invalidate the assay results. Do not substitute or mix Immunostep kit reagents with reagents from other manufacturers.
- Before acquiring samples, it is necessary to ensure that the flow cytometer settings and their compensation are appropriate.
- Keep kit components away from direct light exposure during the protocol. Fluorescently conjugated antibodies and microspheres are sensitive to light.
- Samples should be treated in the same way as those that could transmit infections. Appropriate methods of handling should be available.
- Reagents must not be used if the packaging shows clear evidence of deterioration.
- Wear personal protective equipment for sample handling. Wash hands properly after handling specimens. All procedures should be carried out in accordance with approved safety standards.

⚠ The reagents in this kit include substances of human origin. Although materials of human origin have been tested and found negative for hepatitis B surface antigen (HBsAg), hepatitis C and human immunodeficiency virus, all materials and patient samples should be handled and discarded as potentially infectious using safe laboratory procedures.

⚠ Analyse the colour of the cerebrospinal fluid. Normal CSF is crystal clear, but as a result of trauma, the CSF will appear pink, with a content of more than 400 red blood cells/µl. In this case it may be necessary to perform a dilution for analysis according to section 7.1 of this datasheet.

7. SAMPLE COLLECTION

For multiparameter CMF analyses, collect ≥2 ml of CSF fluid by LP. Refrigerate the sample between +2 and +8°C. The sample must be processed up to 1 hour after LP, otherwise it must be stabilized3 due to the cytotoxic effects of CSF on leukocytes.

In the specific case of having to ship the sample between laboratories or to a central laboratory, it is necessary to stabilize the sample directly at the time of collection, and there are several commercial solutions available on the market, which preserve the cells for a few days (e.g. 48 h). When the stabilized sample arrives at the laboratory, measure and record the volume of CSF received. Do not forget to subtract the volume of stabilizer solution used.

7.1 Sample preparation

When the sample, with or without stabilizer, arrives at the laboratory for analysis, measure and record the volume of CSF received. Do not forget to subtract the volume of stabilizer solution used. Then add 2 ml of filtered/sterile saline buffer ([PBS]; pH 7.4) to the sample and centrifuge 5 min. 540 g and remove the supernatant very carefully, avoiding cell loss, and resuspend the cell pellet in 300 µl of filtered/sterile [PBS]; pH 7.4. For the staining of the sample and the combination of antibodies to be used, it is recommended to follow the EuroFlow recommendations[8-9]. After staining, add 2 ml of FACS lysing solution (BD Biosciences) and incubate for 5 min at room temperature (RT). After incubation, centrifuge the samples for 5 min at 540 g and collect the lysis supernatant in a separate tube, while the cells remaining in the centrifuged tube are resuspended in 50 µl [PBS]; pH 7.4 filtered/sterile, to continue with the cytometer acquisition protocol, according to EuroFlow recommendations[8-9].

The lysis supernatant is the type of sample that can be analysed by this kit, as it contains the analyte (Hb) that allows estimation of the degree of CSF contamination with PB.

The lysis supernatant can be used immediately or can be frozen (-20°C) for up to 6 months for further use with the kit.

At the time of setting up the kit, a visual analysis of the colour of the sample is recommended, in order to determine whether it will be necessary to dilute the sample, using the FACS lysing solution. For this purpose, a guide is attached that allows, depending on the colour, to estimate the dilution of the sample, avoiding the "hook effect" that occurs in samples with high concentration of the analyte and facilitating that the fluorescence fits within the range of interpolation of the calibration curve (Fig. 1).

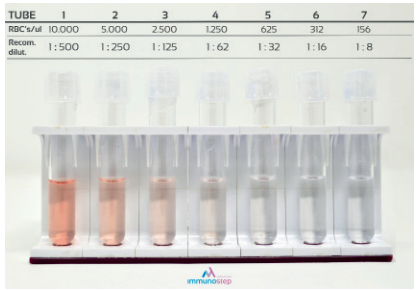

Figure 1: Indicative colour guide corresponding to lysis supernatant sample with different concentrations of RBCs and the suggested dilution that should be used.

Once the dilution to be used has been decided, dilute the samples in FACS lysing solution and mix with a vortex agitator. Diluted samples should be assayed within 2 hours.

8. PREPARATION OF REAGENTS

Temper the reagents at +18°C to +24°C (room temperature) for 30 minutes.

The wash buffer included in the kit is a 10X concentrate. If crystallisation is observed in the concentrated buffer during storage, warm to 37°C and shake well before dilution. To carry out the dilution, the amount required for the assays is removed from the concentrate bottle and diluted 1:10 in PBS, pH 7.4.

Reconstitute standard and positive control in 200 and 100 µl of distilled H2O respectively.
Label as many 12x75 mm polystyrene round-bottomed tubes (cytometer tube) as there are samples, controls, calibration beads and dilutions of the calibration curve to be tested. It is recommended to prepare replicates of each sample, standard and controls.

9. ASSAY PROCEDURE

The protocol can be carried out in a cytometer tube (12x75 mm) (ANNEX I).

1 Preparation of capture beads with the simple	Resuspend the vial of capture beads by vortexing for approximately 20 seconds. To the tubes labelled sample, controls and standard curve, add 5 µl of the capture beads. Then add 45 µl of the HeMoStep incubation buffer.
2 Preparation of controls	The positive (+) control is supplied lyophilized and must be reconstituted immediately before use. Open the vial of the lyophilized positive (+) control and reconstitute with 100 µl H_2O and mix by pipette, do not vortex, then allow to equilibrate for 15 min at RT. Once equilibrated add 50 µl respectively of the positive control (+) and the negative control (FACS lysing), to each of the tubes labeled as such above. Vortex for about 20 seconds. Continue with step 5.
3 Preparation of calibration beads	Resuspend the vial of calibration beads by vortexing for approximately 20 seconds. Add 5 µl of the calibration beads to the tubes listed above. Then add 45 µl of the HeMoStep incubation buffer. It is not necessary to add sample or any control to this tube. The procedure is continued in step 7.
4 Preparation of the standard curve	The standard is supplied in lyophilized form and must be reconstituted immediately prior to use. The kit contains sufficient standard to perform two standard curves. See section 10 of this document for more information. Continue to step 5.
5 Sample incubation	Incubate the tubes (sample, controls, calibration beads and standard curve tubes) for 30 minutes at room temperature, in the dark and under shaking.
6 Washing	After incubation, wash the sample (Hb attached to the beads) once using 1 ml (tube) of 1X wash buffer (see section 8 - Preparation of reagents) for each wash. Leave the wash buffer in each tube for 30 to 60 seconds per wash. Subsequently, collect the magnetic beads by placing the tubes in a magnetic rack and incubating for 5 minutes. The beads can also be collected by centrifugation at 2500xg for 5 minutes. Remove the supernatant from the tubes by manual decanting or by aspiration if centrifugation is used. Take care not to disturb the microspheres and make sure to leave a minimum volume of 50 µl and a maximum of 85 µl of supernatant in the tube.
7 Conjugate incubation	Add 10 µl of the fluorescently conjugated detector antibody into the cytometer tubes. Vortex for about 20 seconds and incubate for 15 minutes at room temperature, in the dark and shaking.
8 Washing	After incubation, wash once as described above (step 6).
9 Measuring	Resuspend the sample in 200 µl of PBS and acquire in a flow cytometer. Store protected from light for a maximum of 30 min at 2-8°C, until acquisition on the cytometer. See point 11 of this document for more information on the analysis and acquisition strategy.

10. PREPARATION OF THE STANDARD CURVE

From the standard of known concentration included in the kit, prepare a 13-point serial dilution (1:2) (Fig. 2), using FACS lysing as the solution.

1 Reconstitution of the freeze-drying	Open the vial of the Lyophilized standard and reconstitute with 200 µl H_2O and mix with the pipette, do not vortex, then allow to equilibrate for 15 min at RT. Finally transfer the reconstituted standard to a 1,5 ml eppendorf tube. This standard is sufficient to generate two standard curves at different times or two replicates in parallel.
2 Perform serial dilutions	Label 13 1.5 ml eppendorf tubes and arrange them in the following order: 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, 1:1024, 1:2048 y 1:4096. Transfer the reconstituted standard to the 1.5 ml eppendorf tube, labelled 1:1 and pipette 100 µl of FACS lysing solution into each of the other labelled tubes (1:2 - 1:4096). Then carry out serial dilutions by transferring 100 µl from the upper standard tube (1:1) to the 1:2 dilution tube and mix well using the pipette. Continue serial dilutions by transferring 100 µl from tube 1:2 to tube 1.4 and so on up to tube 1:4096. Mix thoroughly using the pipette, do not vortex.
3 Transfer to cytometer tubes	Once the serial dilutions have been performed, transfer 50 µl of each of the dilutions to the 12x75 mm tubes (cytometer tube), previously labelled (point 8-preparation of reagents) and already containing 50 µl of the capture beads. Remember that it is recommended to make two replicates of the standard curve. Prepare a 12x75 mm tube containing only FACS lysing solution corresponding to the 0 ng/ml concentration or Non-Specific Binding (NSB) and the negative control. Proceed to step 5 - "sample incubation" of the test procedure, paragraph 9 of this document.

The concentrations (ng/ml) of the analyte (Hb) for each standard dilution as well as an outline of the serial dilution (1:2) of the standard included in the kit for the standard curve assembly are shown in ANNEX II.

With the use of calibration beads it is not necessary to generate a calibration curve every test day.

It will only be necessary to generate a new standard curve in the following cases:
- When using a reagent kit from a new batch.
- When the flow cytometer configuration has been modified.

See point 12 of this document for more information.

11. CYTOMETER ASSAY ACQUISITION AND ANALYSIS STRATEGY

A proper bead population selection strategy has to allow for the removal of doublets and debris, contributing to the correct identification of the bead population.

11.1 Strategy for analysis of samples, controls and calibration curve

A first screening step of the bead population on the FSC-H/FSC-A pattern is recommended to remove doublets (A), followed by a screening of the bead population on the SSC-A/FSC-A dot plot to remove residual dirt and reduce background (B), allowing the correct identification of the bead population on a dot plot for any of the following channels PerCP/APC, PerCP-Cy5/APC or PerCP-Cy5.5/APC (C).

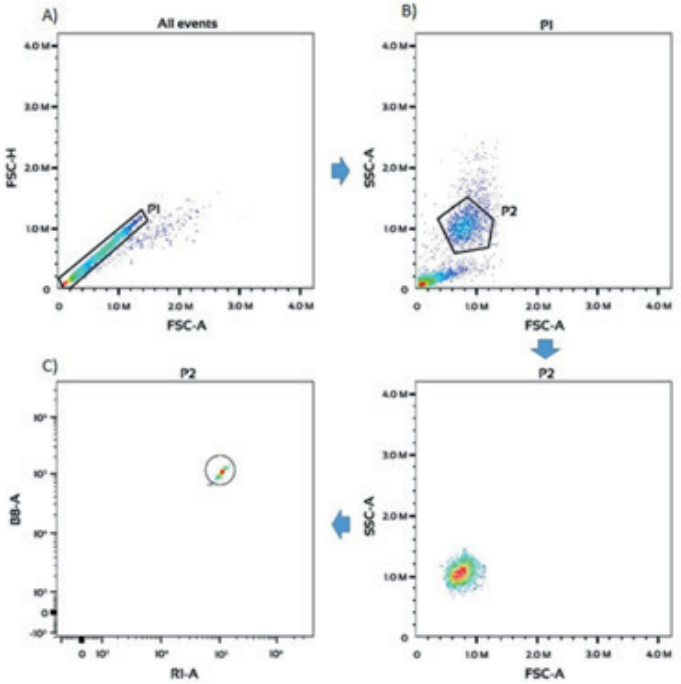

Figure 2: Analysis strategy for bead population selection in the FSC-H/FSC-A (A); SSC/FSC (C) and PerCP/APC (C) dot plots.

11.2 Calibration bead analysis strategy

As in the previous point, a first step of selecting the bead population on the FSC-H/FSC-A dot plot to remove doublets is recommended (A), followed by a selection of the bead population on the SSC-A/FSC-A dot plot to remove residual dirt and reduce background (B), allowing the correct identification of the two calibration bead populations on a dot plot for any of the following channels PerCP/APC, PerCP-Cy5/APC or PerCP-Cy5. 5 / APC (C). The two populations of calibration microspheres have different MFIs for the PE fluorescence channel, the MFIs of the two populations identified as "bottom" and "top" being the parameters (a) and (d) of the standard curve respectively. See section 12.2 of this document for more information.

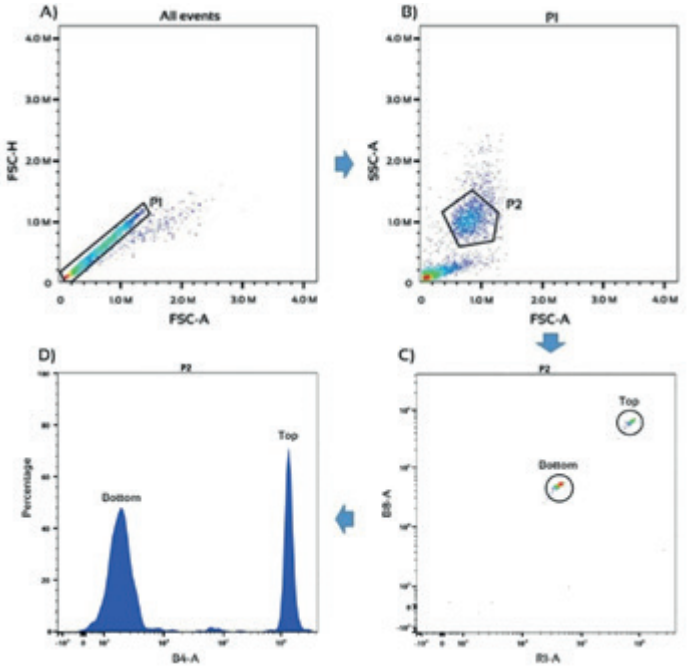

Figure 3: Analysis strategy for the selection of the 2 calibration bead populations in the FSC-H/FSC-A (A); SSC/FSC (C), PerCP/APC (C) and PE histogram (D) dot plots.

12. ANALYSIS OF RESULTS

12.1 Generation of the standard curve

The quantitative assay uses a four-parameter logistic function (4PL) to convert the MFI signal into concentrations.

The first step is to generate the standard curve by using software capable of generating a standard curve fitting this type of regression (Fig. 4). An alternative is to construct a standard curve by plotting the MFI for each standard on the linear (y) axis against the concentration on a logarithmic (x) axis (X=Log(X)) and plot the best-fit curve through the points on the graph. Do not include NSB in the standard curve.

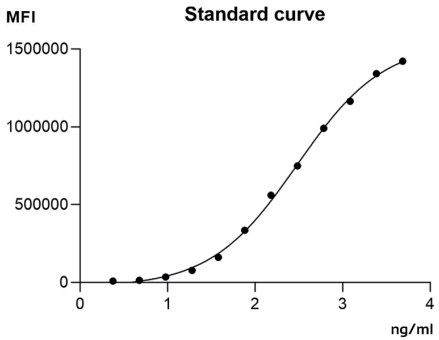

Figure 4: Curva patrón modelo, sólo a modo de orientación. Se debe de construir una curva patrón por lote de kit empleado.

12.2 Calibration of the standard curve

The equation model of a 4PL logistic function is as follows:

$$y = d + \frac{a-d}{1+\left(\frac{x}{c}\right)^{b}}$$

(x) = the independent variable and (y) = the dependent variable.

The 4 estimated parameters consist of the following:
(a) = the minimum value that can be obtained (i.e. what happens at concentration 0)
(d) = the maximum value that can be obtained (i.e., what happens at infinite concentration)
(c) = the inflection point (i.e. the point on the S-shaped curve halfway between a and d)
(b) = Hill slope of the curve, which is related to the slope of the curve at point (c).
(These parameters are calculated by the software we are using).

And they describe a sigmoid pattern curve as shown below (Fig. 5):

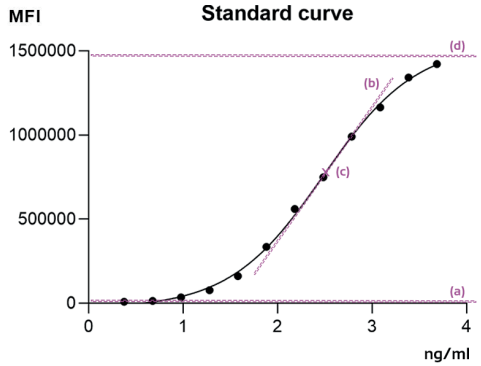

Figure 5: Sigmoid shape curve corresponding to the equation of the four-parameter logistic regression model (4PL).

Whereas the rearranged equation to solve for (x) is:

$$x = c\left(\frac{a-d}{y-d}-1\right)^{\frac{1}{b}}$$

Parameters (b) and (c) define the shape of the curve and parameters (a) and (d) define the position of the curve and the units of (y). If the flow cytometer is properly maintained and calibrated the shape of the standard curve is stable and therefore it is possible to calibrate this non-linear 4PL function with only two calibrators and the information corresponding to the curve shape parameters (b) and (c). The calibration beads, specific to each kit lot, are two populations of beads corresponding to curve parameters (a) and (d). These beads must be acquired in each test run, which allow to check that your MFI values are within a 95% confidence interval (CI), enabling the use of the standard curve without the need for calibration, or if they are outside this 95% CI, they will permit to fit the position of the curve, and it is not necessary to generate a standard curve for each assay. See point 10 of this document for more information.

The curve can only be used to calculate concentrations for MFI signals within (a) and (d). Samples outside the range determined by (a) and (d) cannot be calculated.

12.3 Goodness-of-fit analysis of the standard curve

To analyse how well the data set fits the generated standard curve, in addition to evaluating the R2 value, it is recommended to back-calculate the standard and the recovery of the positive control, because even if the R2 value is very high (>0.99), the precision of the fit determined by the recovery of the standards may indicate otherwise.

12.3.1 Back-calculation of the standard

This involves calculating the concentrations of each of the dilutions of the standard that give rise to the standard curve after the regression has been completed and then comparing them with the actual concentration value using the formula:

[obs] / ([exp]) x 100

[obs] = observed concentration
[exp] = expected concentration

This method provides information on the relative error in the sample calculation, with each standard most desirably falling between 70 and 130% of the true value, although tighter ranges can be used if greater accuracy is desired. Thus, results for samples outside this range may not be accurate.

12.3.2 Recovery of positive control

This method incorporates variables in the preparation of the assay, as well as regression analysis, allowing the overall accuracy of the assay to be evaluated. For this purpose, the positive control with known concentration of the analyte is used and analysed to determine the similarity between the calculated concentration value and the true value (CoA). The result is evaluated in the same way as the recovery of the standards, using the formula: [obs]/([exp]) x100. A recovery value between 80 and 120 % is considered acceptable.

12.4 Calculation of the analyte concentration in the samples

Calculate the logarithmic concentration of Hb by interpolating the MFI values of the samples on the standard curve. Then calculate the antilogarithm ($X = 10^X$) of the value resulting from the interpolation to obtain the concentration value of [Hb] (Fig. 6). If the samples have been diluted, the concentration obtained from the interpolation with the standard curve and its antilogarithm transformation must be multiplied by the dilution factor.

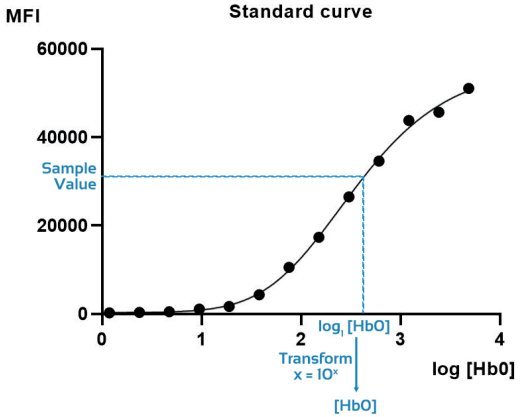

Figure 6: Example of interpolation of the MFI result for a sample on the standard curve and calculation of the [Hb] concentration in the sample.

12.5 Interpretation of results

Traditionally, CSF contamination with PB due to traumatic LP has been expressed in [RBC/µl]. Thus, for a better interpretation and standardization of the results, it is recommended to express the degree of contamination in [RBC/µL].

For this purpose, from the result of the [Hb] concentration of the lysis supernatant (point 12.3) and using the CSF volume recorded (point 7.1) and the values of haemoglobin concentration (g/dL) and erythrocyte count (RBC no. x10⁶/µl) from the patient's SP or from the CoA, in case no blood count is available, calculate the [RBC/µl] concentration in the CSF, following the workflow described in ANNEX III.

Likewise, as a result of the calculation of the dilution factor of PB in CSF, described in APPENDIX III, it is also possible to estimate the number of leukocytes (WBC) present in the CSF sample from the SP contamination. See ANNEX III for more information.

12.6 Expected values

The study was conducted on 105 CSF samples, which were free of contamination by visual inspection. The sample analysed was the lysis supernatant resulting from sample processing and the following results were obtained:

	All samples			Traumatic lumbar puncture samples)		
	Mean	95th percentile	n	Mean	95th percentile	n
	1201	34,47 - 2368	105	1845	288 - 3402	34
RBC/ µl*	38	9 - 67	82	101	21 - 182	29

**Calculated in accordance with point 12.4 of this document.*

Each laboratory should investigate the expected values for its own sample population and, if necessary, determine its own reference ranges.

13. PERFORMANCE CHARACTERISTICS

13.1 Detection capability

The detection limit study, sometimes also known as analytical sensitivity, was performed according to the EP17-A2 guideline (CLSI). Assessment of Clinical Detection Capability. The result of this study is expressed as [Hb ng/ml].

- The limit of detection (LoD) of the test or lowest detectable concentration of the analyte by the test is [3.15 ng/ml] of Hb.
- The limit of quantification (LoQ) of the test or lowest analyte concentration that can be accurately quantified is [3,23 ng/ml] of Hb.

13.2 Precision

For the intra-laboratory precision study, four (4) samples were selected (negative, LoD, positive control and positive sample) and following CLSI recommendations (EP05-A3), Evaluation of Quantitative Measurement Precision. A 20 x 2 x 2 experimental design was selected, consisting of a study lasting at least 20 days, with two runs for each day the assay is conducted and with two replicates per sample tested in each run. The study was conducted on a single instrument. The results were as follows:

		Within-run	Between-run	Between-day	Within-lab
Pos	n	3,74			
	Mean value (ng/ml)	92,03			
	SD (ng/ml)	3,74	13,75	3,74	13,75
	CV (%)	4,06%	14,95%	4,06%	14,95%
Control +	n	78			
	Mean value (ng/ml)	10,85			
	SD (ng/ml)	0,35	1,80	0,35	1,80
	CV (%)	3,28%	16,85%	3,28%	16,85%
LoD	n	78			
	Mean value (ng/ml)	2,90			
	SD (ng/ml)	0,03	0,11	0,03	0,11
	CV (%)	0,87%	3,86%	0,87%	3,86%
Neg	n	80			
	Mean value (ng/ml)	2,41			
	SD (ng/ml)	0,02	0,03	0,02	0,03
	CV (%)	0,74%	1,14%	0,79%	1,11%

13.3 Reproducibility

Similarly, a reproducibility study was carried out, with a 5x2x2 experimental design, consisting of a 5-day study, in two laboratories and two replicates of each sample. The study was carried out to see if there are differences between equipment, with the following results:

		Within lab
Positive	n	20
	Mean Value	131,9645973
	SD	37,6628082
	CV	23,19%
LoD	n	14
	Mean Value	2,756914985
	SD	1,513798652
	CV	29,02%

13.4 Comparing methods

A comparison of methods between the absolute neutrophil granulocyte count by CMF (x) and the HeMoStep kit (y) was performed on a total of 29 samples classified as traumatic LP according to the reddish colour of the CSF and/or the observation of a red coloured cell pellet after centrifugation of the sample, showing a good direct linear correlation ($r = 0.9081$; $p: <0.0001$) between the variables n° of granulocytes and n° of RBC calculated with each method.

13.5 Analytical specificity

Pathological CNS bleeds result in extravasation of RBCs that lyse into the CSF, with eventual catabolism of haemoglobin to bilirubin[10]. The latter occurs approximately 12 hours after a haemorrhage and persists for two weeks. For this reason, the possible cross-reactivity that high bilirubin levels could have on the performance of the assay was analysed. For this purpose, CSF samples were selected and artificially contaminated with SP and spiked with 0.4 mg/ml bilirubin. The samples were then processed and analysed according to the kit instructions and in no case were significant differences observed between bilirubin-enriched and non-enriched samples.

Additionally, the possible interferences of commercially available CSF stabilising solutions such as TransFix (Cytomark) and Streck Cell Preservative (Streck Inc) were also analysed. CSF samples were selected and artificially contaminated with PB, then diluted with TransFix at 1:10 and 1:20 and Streck Cell Preservative using a 1:10 dilution, and left to stabilise overnight (O/N) at 4°C. All cases were compared to contaminated CSF without interferents or stabilisers. Samples were tested according to the kit instructions and in no case were significant differences between samples found.

14. LIMITATIONS OF THE PROCEDURE

- The results should be evaluated in combination with other diagnostic procedures
- Results outside the range determined by the standard curve. Refer to section 12.2 of this document for further information.
- The results of the assay depend on the sample collection and processing procedures having been performed properly.
- For the conversion of the [Hb - ng/ml] result of the lysis supernatant to [RBC/µl] in the CSF, it is assumed that the volume of FACS lysing used is 2000µl. See ANNEX III for more information.

15. BIBLIOGRAPHY

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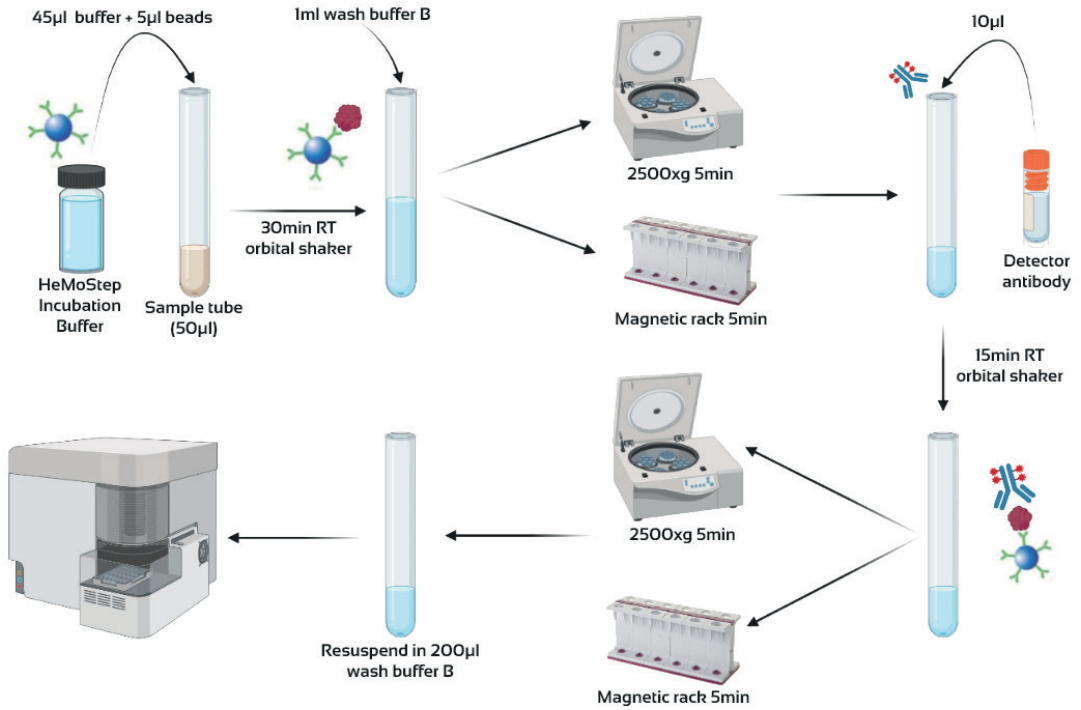
16. EXPLANATION OF SYMBOLS

Σ	Content is sufficient for <n> analysis
REF	Reference or product identifier
CE	CE labeling
IVD	*In vitro* diagnostic device
	Manufacturer
	Expiry date
LOT	Batch code
	Follow the instructions for use
	Preservation from x°C to y°C
	Contents of each test
	Pay attention to
	Biohazards
BEADS	Capture beads
WASHBUF 10X	Wash buffer 10x
CNTRL+	Positive control
CAL	Calibration beads
STD	Standard of known concentration
CONJ IgG	Fluorescently conjugated detector antibody

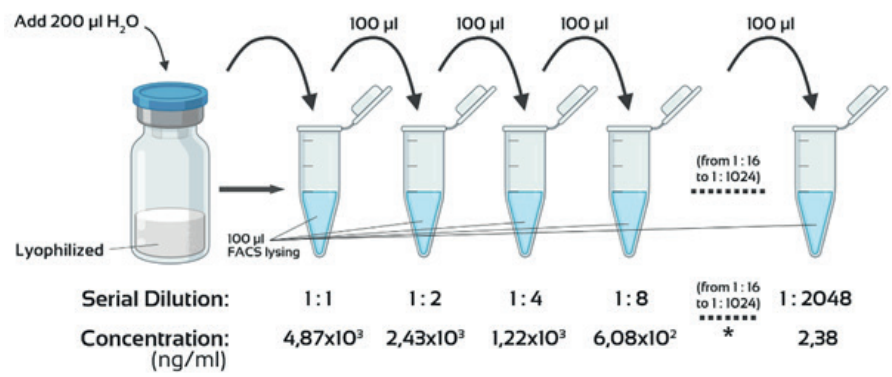
17. DATOS DEL FABRICANTE

IMMUNOSTEP S.L.
Address: Avda. Universidad de Coimbra, s/n
Cancer Research Center (C.I.C)
Campus de Unamuno
37007 Salamanca (Spain)
Telf./fax: (+34) 923 294 827
E-mail: info@immunostep.com
www.immunostep.com

ANNEX I. Cytometer tube workflow

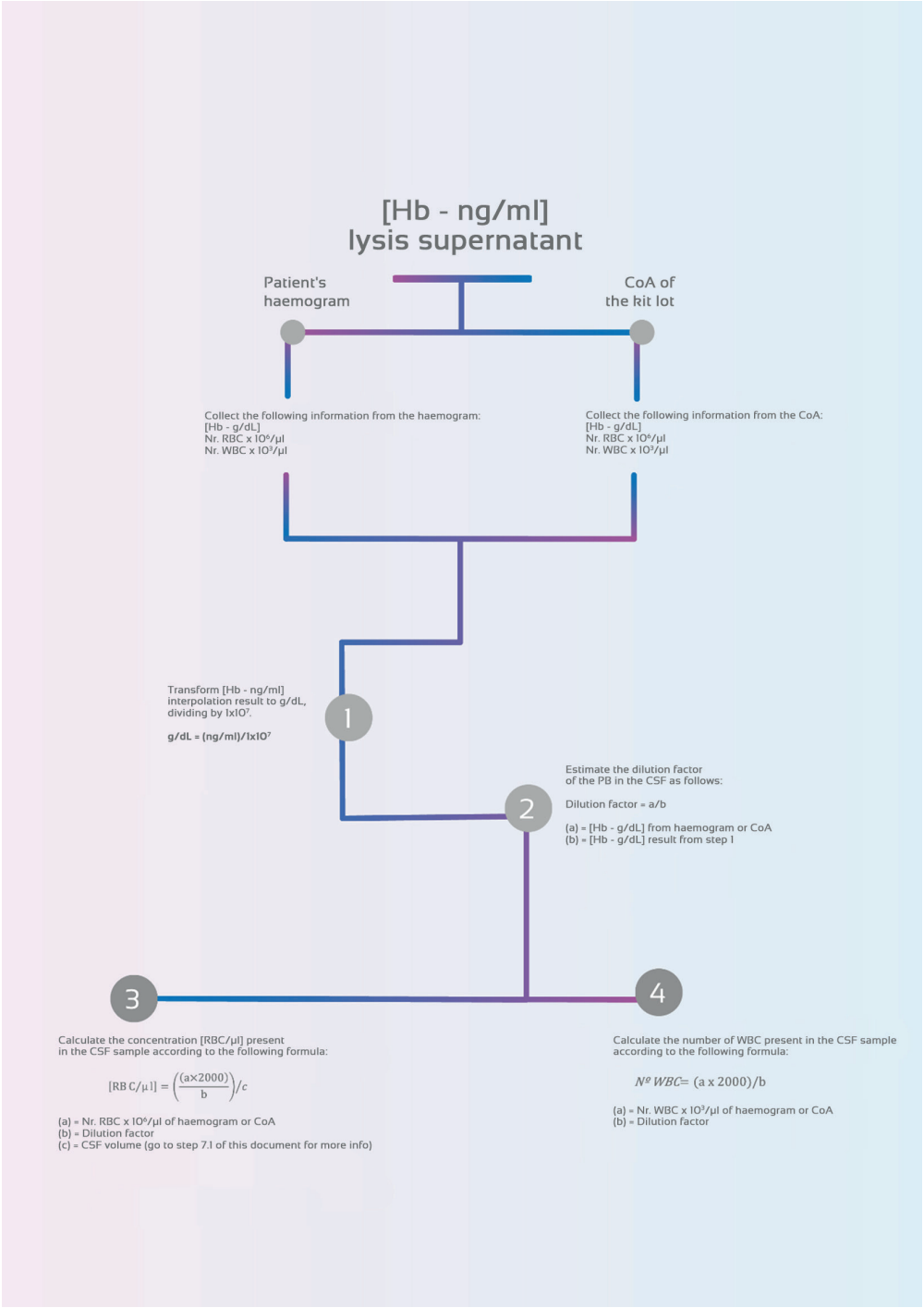


ANNEX II. Serial dilution (1:2) of the standard for the construction of the standard curve



*From 1:16 to 1:1024

Serial Dilution	Concentr. (ng/ml)
1:16	38,0
1:32	19,0
1:64	9,50
1:128	4,75
1:256	2,38
1:512	9,50
1:1024	4,75
FACS lysing (B_0)	0,00


[Hb - ng/ml]
lysis supernatant
Patient's haemogram
CoA of the kit lot
Collect the following information from the haemogram:
[Hb - g/dL]
Nr. RBC x 10⁶/µl
Nr. WBC x 10³/µl
Collect the following information from the CoA:
[Hb - g/dL]
Nr. RBC x 10⁶/µl
Nr. WBC x 10³/µl
Transform [Hb - ng/ml] interpolation result to g/dL, dividing by 1x10⁷.
g/dL = (ng/ml)/1x10⁷
1
2
Estimate the dilution factor of the PB in the CSF as follows:
Dilution factor = a/b
(a) = [Hb - g/dL] from haemogram or CoA
(b) = [Hb - g/dL] result from step 1
3
4
Calculate the concentration [RBC/µl] present in the CSF sample according to the following formula:
$[\mathrm{RBC}/\mu\mathrm{l}] = \left(\frac{(a \times 2000)}{b}\right)/c$
(a) = Nr. RBC x 10⁶/µl of haemogram or CoA
(b) = Dilution factor
(c) = CSF volume (go to step 7.1 of this document for more info)
Calculate the number of WBC present in the CSF sample according to the following formula:
$Nº\ WBC = (a \times 2000)/b$
(a) = Nr. WBC x 10³/µl of haemogram or CoA
(b) = Dilution factor