LIOFeron® TB/LTBI

Instructions for use 2 Component Kit

Component 01

HUMAN BLOOD STIMULATION TUBES

Σ	22 Tests	REF	LIO-Feron 01_22
Σ	2 x 22 Tests	REF	LIO-Feron 01_44

Component 02

HUMAN IFN-γ ELISA

Σ	22 Tests	REF	LIO-Feron 02_22
Σ	44 Tests	REF	LIO-Feron 02_44

Interferon Gamma (IFN-γ) Release Assay (IGRA) in human blood for diagnosing Latent TB Infection (LTBI). As for other IGRA tests on the market, this test may react positive in TB patients also, but it cannot differentiate between LTBI and active TB. For professional in vitro diagnostic use only! Not for personal use!

IVD For in vitro diagnostic use

CE

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LIONEX Diagnostics and Therapeutics

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Intended use

The **LIOFeron®TB/LTBI** is a cytokine release assay for quantitative determination of Interferon Gamma (IFN-γ) produced by human blood cells stimulated with *Mycobacterium tuberculosis* antigens. Thus, the test is useful for the diagnosis of Latent TB Infection (LTBI). As for other IGRA tests on the market, this test may react positive in TB patients also, but it cannot differentiate between LTBI and active TB.

The **LIOFeron®TB/LTBI** is a 2 component kit consisting of **HUMAN BLOOD STIMULATION TUBES** and **HUMAN IFN-γ ELISA**. The test is intended for professional in vitro diagnostic use. The test is NOT intended for personal use.

Introduction / Field of application

Human tuberculosis (TB) has become a global disease with its re-emergence in the Western countries in the last decades. TB is predominantly a disease of the respiratory tract but can also affect other organs. People who are suffering from active pulmonary TB are highly infectious. The spread of TB takes place by coughing and sneezing. LTBI is not infectious and occurs when the individual's immune system has controlled the primary TB infection. Latent infection can remain throughout one's life and can be reactivated when the individual's immune system is weakened, for example, in old age, cancer or due to infection with other diseases.

According to the latest estimates of the World Health Organization (WHO), TB is one of the top ten causes of death worldwide and the leading cause from a single infectious agent. In 2017, TB claimed an estimated 1.3 million deaths (range, 1.2-1.4 million) among HIV-negative people, and there were an additional 300000 deaths from TB (range, 266000-335000) among HIV-positive people. There were an estimated 10.0 million new cases of TB (range, 9.0-11.1 million)[24]. Drug resistance to HIV-treatment and appearance of multiple-drug resistance (MDR) and of Extensively Drug Resistance (XDR) strains of *M. tuberculosis*, the causative agent of human TB is steadily leading to a hopeless situation as far as the therapy is concerned. According to WHO, globally, 160684 cases of MDR/RR-TB were detected and notified in 2017[24].

To make things worse, there is no effective vaccine available against HIV and TB. *M. bovis* BCG, the only vaccine available against TB, has shown highly variable efficiency and has been very often ineffective[25].

Diagnosis of infections with *M. tuberculosis* can be very helpful in decision making considering treatment options. Human blood levels of various cytokines have proven to be of significant use in diagnosing infections with *M. tuberculosis*.

T-lymphocytes from people infected with *M. tuberculosis* recognize mycobacterial antigens and provoke cytokine production, e.g. IFN-γ[9, 12-14]. The quantitative determination of IFN-γ forms the basis of the Interferon-Gamma (IFN-γ) Release Assay (IGRA), called **LIOFeron®TB/LTBI** and can be useful of diagnosis of both, active TB and latent TB but the test cannot differentiate between LTBI and active TB.

IGRAs are widely used to determine IFN-γ-response of human Peripheral Blood Mononuclear Cells (PBMCs) to active- and latent tuberculosis by using recombinant antigens of *M. tuberculosis*. **The WHO has endorsed IGRAs as the preferred test for the detection of latent TB Infection (LTBI) in BCG-vaccinated persons in low-burden TB countries.**

LIOFeron®TB/LTBI has two different TB antigen tubes, TB A and TB B. TB A contains ESAT6, CFP10 and TB 7.7 antigens containing CD4 epitopes and commonly used widely for IGRA tests[2-4, 7, 10, 11, 17, 19, 20] and which cover all peptides pertaining to these antigens and TB B contains a proprietary antigen alanine dehydrogenase of LIONEX with CD8+ epitopes[4, 18] but which also contains peptides for detecting CD4 T cell response, our unpublished results.

No matter whether one uses peptides or full-lebgth antigen, what is crucial and relevant to the diagnosis is a comparative clinical study where Quantiferon (Qiagen) and Lioferon (LIONEX) are compared using the same blood sample. This was done in the study which has been published showing very strong concordance between Qiantiferon and Lioferon . This had proven that it is not essential to use peptides in such tests, full-length antigens are effective. In addition, one should consider the fact that such tests of several manufacturers which contain full-length proteins instead of peptides have been recommended for diagnosis by the World health organisation (WHO). Presentation of full-length antigen is a natural way.

Tube labelled NC means Negative Control containing no antigen (NIL). Tube labelled PC means Positive Control containing a mitogen PHA.

Principles of the test

The **LIOFeron®TB/LTBI** consists of two main components, the **HUMAN BLOOD STIMULATION TUBES** and the **HUMAN IFN-γ ELISA**. The Test is a cytokine release assay based on the fact that cells from human blood will secrete IFN-γ when exposed to specific *M. tuberculosis* antigens.

The **HUMAN BLOOD STIMULATION TUBES** contain a positive control tube, a negative control tube and two TB antigen tubes for each sample. The human blood sample (in Li-Heparin blood collection tubes; not provided in the kit) is taken by venipuncture and 1 mL each is pipetted into negative-, positive- and TB antigen stimulation tubes provided in the kit. The tubes are gently mixed by shaking upside down and placed into a 37°C incubator overnight. Next, the clear supernatant (plasma) is carefully removed and analyzed using **HUMAN IFN-γ ELISA** which quantitates the amount of IFN-γ produced in response to the specific antigens from *M. tuberculosis*. These special antigens are distinguishable from those present in BCG and most other non-tuberculous mycobacteria (NTM).

The **HUMAN IFN-γ ELISA** is based on the principle of the enzyme immunoassay (EIA). Monoclonal anti-human IFN-γ specific antibodies are bound on the surface of the microtiter wells as capture reagent.

The plasma samples from the HUMAN BLOOD STIMULATION TUBES are pipetted into the wells of the microtiter plate in parallel to the standards. Then a biotin-conjugated secondary antibody (Detection Antibody Solution) is pipetted into the wells and the microtiter plate is incubated. If IFN-γ is present in the sample, it will bind to immobilized antibodies on the microtiter plate. After the incubation step, the plate is rinsed with diluted wash solution to remove unbound material. Following the washing step, the Conjugate Solution is added and the plates are incubated. After a final washing step, the Substrate Solution is pipetted, inducing the development of a blue dye in the wells. The colour development is terminated by the addition of a Stop Solution which changes the colour from blue to yellow. Wells containing no IFN-γ remain colourless. The resulting colour is measured by an ELISA reader at the wavelength of 450 nm. The concentration of IFN-γ is directly proportional to the intensity of the colour. Measuring each set of samples in parallel to the standards allows quantitative determination of the IFN-γ concentration in each sample

Supplied materials

Table 1: Components of the **HUMAN BLOOD STIMULATION TUBES**:

HUMAN BLOOD STIMULATION TUBES:				
Cap colour		Σ / REF	22 Tests **LIO-Feron 01_22**	2x 22 Tests **LIO-Feron 01_44**
■	Positive control (ready-to-use) contains Li-Heparin and mitogen (black cap)	PC	22 tubes	2x 22 tubes
□	Negative control (ready-to-use) contains contains Li-Heparin (white cap), contains no antigen (NIL)	NC	22 tubes	2x 22 tubes
■	TB antigen A (ready-to-use) contains contains Li-Heparin and ESAT6, CFP10 and TB 7.7 antigens (green cap)	TB A	22 tubes	2x 22 tubes
■	TB antigen B (ready-to-use) contains Li-Heparin and LIONEX antigen with CD8+ epitope (blue cap)	TB B	22 tubes	2x 22 tubes

Table 2: Components of the **HUMAN IFN-γ ELISA**:

HUMAN IFN-γ ELISA:				
Cap colour		Σ / REF	22 Tests **LIO-Feron 02_22**	2x 22 Tests **LIO-Feron 02_44**
	Microtiter plate (ready-to-use) with monoclonal anti-human IFN-γ capture antibody, sealed in aluminium pouch with desiccant, 12 x 8 well strips	MTP	1 x	2 x
■	Recombinant human IFN-γ standard (12.5 IU, lyophilised) (red cap)	STA	2 x	4 x
■	Detection Antibody Solution (ready-to-use) with monoclonal anti-human IFN-γ detection antibody, biotin-conjugated, contains casein (green cap)	DET	7 mL	14 mL
■	Conjugate Solution (ready-to-use) with streptavidin, HRP-conjugated (yellow cap)	CON	14 mL	24 mL
■	Substrate Solution (ready-to-use) contains 3,3',5,5'-Tetramethylbenzidine* (TMB) (black cap)	TMB	14 mL	24 mL
□	Stop Solution (ready-to-use) contains H_2SO_4* (white cap)	STO	7 mL	14 mL
■	Wash Buffer (10x concentrate) (black cap)	WB	100 mL	2x 100 mL
■	Incubation Buffer (ready-to-use) contains bovine serum albumin (blue cap)	IB	40 mL	40 mL
	Instructions for use		1 x	1 x

* Refer to section for "*Warnings and precautions*".

Materials needed but not provided

- Disposable gloves; Waste containers for potentially contaminated materials
- Li-Heparin tubes or Li-Heparin tubes without stimulation of intravenous blood into tubes.
- Tubes for plasma collection and dilution (optionally, e.g. sterile 1.5 mL tube)
- Tubes for standard preparation (e.g. sterile 1.5 mL tube)
- Incubator 37°C (± 0.5 °C), CO_2 is not essential
- Centrifuge suitable for blood tubes (RCF range from 2000 to 3000)
- Vortexer
- Deionized or destilled water
- Beakers, flasks and/or graduated cylinders necessary for 1x wash buffer preparation
- Calibrated variable pipets for a volume of 50 µL to 1000 µL with disposable tips
- Microtiter Plate-Washer (recommended) or calibrated multichannel pipets for a volume of 50 µL to 400 µL with disposable tips (optional)
- Timer
- Microtiter Plate-Reader (450 nm)

Test procedure time

The required time to perform the **LIOFeron®TB/LTBI** is shown below.

HUMAN BLOOD STIMULATION TUBES:	Procedure time for human blood sample application requires approx. 10 minutes. Incubation of human blood samples requires 16 to 24 hours (37°C ± 0.5 °C). Procedure time for human blood sample harvesting requires approx. 5 minutes.
HUMAN IFN-γ ELISA:	Approx. 3 hours for one ELISA plate (22 tests). < 1 hour laboratory work required, add approx. 15 minutes for each extra plate.

Warnings and Precautions

For in vitro diagnostic use! Not for personal use!

- Follow the instructions of the test procedure and interpretation of results carefully!
- In accordance with Good Laboratory Practice (GLP), all laboratory devices employed should be regularly checked and calibrated for the accuracy and precision.
- Do not ingest or swallow! Do not eat, drink and smoke in the laboratory! Do not work without wearing protective clothing (disposable gloves, safety glasses and lab coat)! Avoid the contact of kit reagents with skin, eye or mucosa.
- Use all reagents within the expiry period (printed on the labels).
- Bring all test components to room temperature (preferably 15 - 30°C) and invert liquid test components gently before use. Return test components immediately to 2 - 8°C after usage. The test is sensitive to temperatures above 30°C.
- Use only fresh blood samples containing anticoagulants (Li-Heparin). Body fluids other than Li-Heparin human blood are not validated and can yield incorrect results! Store blood samples at room temperature (preferably 15 - 30°C)! Do not store blood samples below 15°C! The blood shall not be used, if it is older than 16 hours after venipuncture.
- We recommend the use of double determinations for standard and blank measurements. In order to measure the samples, single determinations are possible for human plasma samples.
- Do not use reagents from different kit lots or batch codes and do not mix reagents of different kit lots or batch codes.
- Avoid contamination of the reagents. Do not use the same container for several samples!
- Work under sterile conditions to avoid contamination of samples. Avoid the use of turbid samples which may be contaminated with bacteria. Only the clear supernatant shall be used for cytokine measurements. While collecting the supernatant avoid contamination by red blood cells. If necessary, separate the red blood cells from plasma by centrifugation.
- Avoid repeated freezing and thawing of the plasma samples because it could lead to denaturation of the cytokines.

- Before pipetting, mix all reagents thoroughly by gentle tilting or swinging. Vigorous shaking with formation of foam should be avoided. Pipet with constant intervals, so that all wells of the microtiter plate have the same conditions.
- Avoid touching of the inlet of vial screw cap with your fingers (danger of contamination). For single-use only. Do not use if the vials are damaged or open (no screw cap).
- Protect the TMB Substrate Solution from direct sunlight.

For more information, please request the **Material Safety Data Sheets (MSDS)** via E-mail to sales@lionex.de.

ATTENTION:
Handle human blood and plasma as potentially infectious. All kit components should be considered as infectious agents. Decontaminate and dispose remaining kit reagents and human blood samples in accordance with federal, state and local regulations, e.g. by autoclaving or using a disinfecting solution.

Hazard and Precautionary Statements

TMB **Substrate Solution**
3,3', 5,5'-Tetramethylbenzidin (TMB) contains: N-Methyl-2-pyrrolidon. Danger! May damage fertility or the unborn child (H360D).
Dispose the kit components to an approved waste disposal plant.
If exposition occurred: Get medical advice and obtain special instructions before use. Wear protective gloves and lab coat (eye protection, face protection).

STO **Stop Solution**
Contains: Sulfuric acid. Danger! Causes severe skin burns and eye damage (H314). May be corrosive to metal (H290).
Dispose the kit components to an approved waste disposal plant.
If contact with eyes: Rinse cautiously with water for several minutes. Remove contact lenses, if they are present and easy to remove. Continue rinsing.
If contact with skin: Remove immediately all contaminated clothing. Rinse skin with water. Immediately call a poison center or doctor. Wear protective gloves and lab coat (eye protection, face protection).

Sample collection and preparation

The **LIOFeron®TB/LTBI** works best with fresh human blood samples. The blood sample taken from the patient into a tube with L-Heparin or taken directly from the vein with a syringe, butterfly set, etc. is distributed as 1 ml each into the blood stimulation tubes.

Minimum 4.5 mL human blood collected under standard laboratory conditions (aseptically, avoid haemolysis) by using Li-Heparin blood collection tube is needed.

If the application of **HUMAN BLOOD STIMULATION TUBES** cannot be performed immediately after blood sampling from the vein, the whole blood can be stored for up to 16 hours at 15 - 30°C.

Important note on Blood collection and dispensing in the tubes:

1. Whole blood can be collected in standard Lithium Heparin blood collection tubes and shipped to clinical laboratory where one ml blood is added to each tube and incubated.

or

2. Whole blood which is taken with injectors, butterfly sets, etc. can be directly added to the LIOFeron TB/LTBI **HUMAN BLOOD STIMULATION TUBES** provided in the kit followed by incubation because the LIOFERON Blood collection tubes contain Li-Heparin anticoagulant.

Use and storage of stimulated plasma

We recommend to transfer the plasma directly after centrifugation from the tubes to the ELISA plate. Hence, we suggest to perform the **HUMAN IFN-γ ELISA** within a few days after blood stimulation.

If the **HUMAN IFN-γ ELISA** test cannot be performed immediately after stimulation, the tubes can be stored for up to 4 days at 2 – 8°C.

For longer storage separate human plasma from the red blood cells and store at 2 - 8°C (up to 28 days). But this may lead to decrease in the concentration of IFN-γ. If harvested, plasma samples can be stored for extended periods below -20°C. Frozen samples must be thawed prior to testing and well mixed. Avoid repeated freezing and thawing of samples!

Preparation of reagents

WB Wash Buffer (10 x concentrate):

Equilibrate WB wash buffer (10 x concentrate) to room temperature (preferably 15 - 30°C) and invert gently before use. If crystals precipitate during the cold storage, the concentrate should be warmed up at 37°C for 15 minutes.

Before use, dilute with distilled water (1 + 9 volume), e.g. 100 mL WB Wash Buffer (10 x concentrate) + 900 mL distilled water.

STA Recombinant human IFN-γ standard (lyophilised):

Add the volume of IB Incubation Buffer indicated on the label into the vial for reconstitution of lyophilised recombinant human IFN-γ standard to final concentration of 12.5 IU/mL. Mix the stock solution after addition of the Incubation Buffer for 10 seconds (e.g. Vortexer) until the protein is fully solubilized. We recommend to leave the tube for 10 – 30 min at room temperature.
Prepare standard solutions S1 - S4 for standard curve by using the stock solution according to the following pipetting scheme (Table 3 and Figure 1):

Table 3: Preparation of standard solution no. S1 - S4.

Standard solution no.	conc. IFN-γ	Volume of Standard solution	Volume of Incubation Buffer	Total Volume
S1	**4 IU/mL**	80 µL of stock solution	170 µL	250 µL
S2	**1 IU/mL**	50 µL of solution S1	150 µL	200 µL
S3	**0.25 IU/mL**	50 µL of solution S2	150 µL	200 µL
S4 (Blank)	**0 IU/mL**	-	150 µL	150 µL

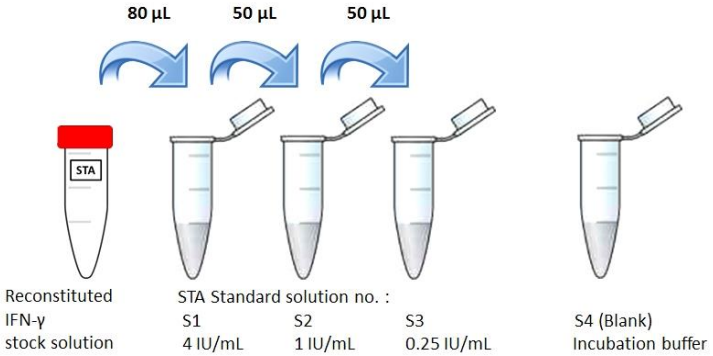

Figure 1: IFN-γ concentrations of the prepared standard solutions S1 – S3.

Stability and storage conditions of reconstituted standard and other reagents

HUMAN BLOOD STIMULATION TUBES

- Store at 2 - 30°C.
- **DO NOT EXPOSE** the test components to temperatures above 30°C.

HUMAN IFN-γ ELISA

- Store at 2 - 8°C.
- **DO NOT FREEZE** the test components.
- **DO NOT EXPOSE** the test components to temperatures above 30°C.
- Protect the TMB Substrate Solution from direct sunlight.

The MTP microtiter plate is sensitive to moisture. After first opening of microtiter plate the stability is given up to 3 months, if the remaining microtiter plate stripes will be stored at 2 - 8°C in an aluminium bag (closed by zipper) with desiccant.

After dilution of the WB Wash Buffer (10 x concentrate) the diluted solution (1x wash buffer) is stable for 1 month at 2 - 8°C. For instructions on how to prepare the 1x wash buffer, please see section "*Preparation of reagents*".

The reconstituted STA stock solution is stable for 2 months at 2 - 8°C. Note the reconstitution date on the label of the STA vial. For instructions on how to prepare the standard solution no. S1 -S4, please see section "*Preparation of reagents*".

Unopened kit components are stable until the expiry date. The expiry date is printed on the labels of each test component and on the outer packaging. Do not use if test components are damaged or open (e.g. no screw cap, aluminium bag damaged). After first opening of liquid components the stability is minimum 3 months, if the bottles are tightly closed after every usage.

NOTE: If precipitates appear in the CON Conjugate Solution, it will not affect the test result. If bubbles are visible in the unused wells, it will not affect the test result.

Test procedure

HUMAN BLOOD STIMULATION TUBES

Test procedure time requires 10 minutes for each sample. Incubation time: 16 - 24 h.

1. Take the required number of **HUMAN BLOOD STIMULATION TUBES** NC, TB A, TB B and PC **from the kit**. Place them in a rack/holder. Label the **HUMAN BLOOD STIMULATION TUBES** appropriately, e.g. by sample number, ID or Barcoad. Remove the lid of each tube by **pulling up the cap (no screwing)** and place them on flat surface.
2. Invert the Li-Heparin blood collection tube with the sample collected several times upside down. Take care that the sample is homogeneous. If a pellet is visible when the tube is turned over, loosen it carefully (e.g. by gently shaking). Add one ml blood sample to each of the four **HUMAN BLOOD STIMULATION TUBES** (NC, TB A, TB B and PC).

3. Mix each **HUMAN BLOOD STIMULATION TUBES** filled with 1 mL Li-Heparin blood 10 times gently upside down. Avoid vigorous shaking, otherwise the blood cells could haemolyse!

4. Immediately place the rack with **HUMAN BLOOD STIMULATION TUBES** filled with 1 mL Li-Heparin blood upright in an incubator at 37°C (± 0.5 °C) for minimum 16 hours. Maximum incubation duration is 24 hours.

5. Finally, take out the rack with **HUMAN BLOOD STIMULATION TUBES** from the 37°C incubator (without shaking!). **It is possible to harvest the plasma without centrifugation but take care to avoid contamination with red blood cells**. The red blood cells accumulate in the gel plug which separates the cells from the plasma.

   Important note: If contamination with red blood cells is observed harvest the human plasma by centrifugation of the tubes for 15 minutes at 2000 to 3000 RCF (g). After centrifugation, avoid pipetting up and down or mixing plasma by any means prior to harvesting. At all times take care not to disturbe material on the surface of the gel.

6. Plasma samples should only be harvested using a pipet. Load directly from **HUMAN BLOOD STIMULATION TUBES** into the **HUMAN IFN-γ ELISA** plate. Transfer 50 µL of the clear supernatant (human plasma) from the HUMAN BLOOD STIMULATION TUBES into wells of microtiter plate and continue with step 5 of “HUMAN IFN-γ ELISA Test procedure”.

   Note: It is possible to use an automated ELISA workstation.

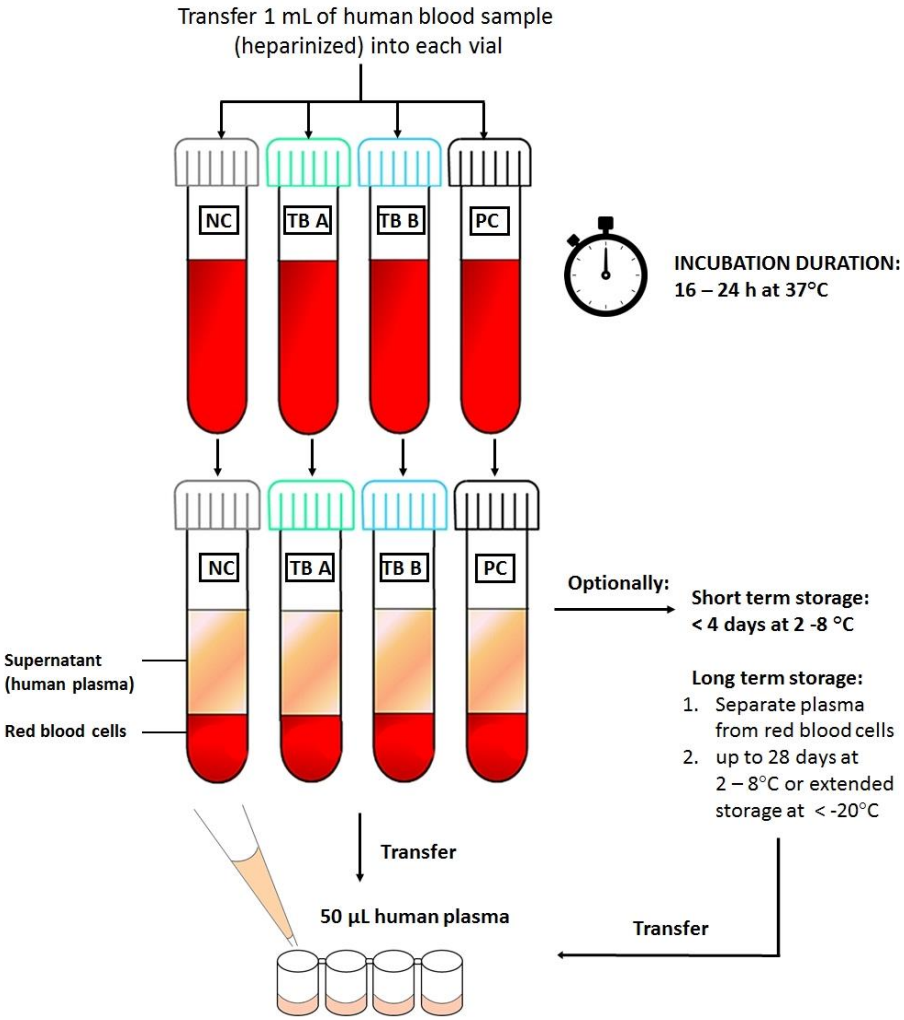

Figure 2: Quick Reference Guide for using **HUMAN BLOOD STIMULATION TUBES**. Transfer the samples (supernatant) by directly pipetting into the ELISA plate. It is also possible to use automatic ELISA workstation. Optionally store at 2 – 8°C or below -20°C as indicated in "Use and storage of stimulated plasma".

HUMAN IFN-γ ELISA

Test procedure time requires 3 hours.

1. Place the prepared wash buffer and the standards from section “preparation of reagent” ready

2. Equilibrate all kit components to room temperature (15 - 30°C, approx. 30 minutes).

3. Remove the MTP microtiter plate from the aluminum pouch and place required microtiter strips in provided holder. Transfer unused microtiter strips back into aluminum pouch.

4. Pipet 50 µL / well of each sample, prepared standard solution no.´s S1 - S3 and IB Incubation Buffer as blank (S4) into the wells. The standards and blank should be measured in duplicate, the samples as single determinations. Samples and standards can be pipetted at any place in the ELISA plate but if pipetted from top to bottom (refer to example shown in Figure 3 below), it will facilitate analysis of the results manually or by a software. Stored plasma samples should be mixed thoroughly before use! Layout can be vertical, horizontal or free plate layout.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1 NC	3 NC	5 NC	7 NC	9 NC	S1		13 NC	15 NC	17 NC	19 NC	21 NC
B	1 TB A	3 TB A	5 TB A	7 TB A	9 TB A	S2		13 TB A	17 TB A	17 TB A	19 TB A	21 TB A
C	1 TB B	3 TB B	5 TB B	7 TB B	9 TB B	S3		13 TB B	15 TB B	17 TB B	19 TB B	21 TB B
D	1 PC	3 PC	5 PC	7 PC	9 PC	S4 (Blank)		13 PC	15 PC	17 PC	19 PC	21 PC
E	2 NC	4 NC	6 NC	8 NC	10 NC	11 NC	12 NC	14 NC	16 NC	18 NC	20 NC	22 NC
F	2 TB A	4 TB A	6 TB A	8 TB A	10 TB A	11 TB A	12 TB A	14 TB A	16 TB A	18 TB A	20 TB A	22 TB A
G	2 TB B	4 TB B	6 TB B	8 TB B	10 TB B	11 TB B	12 TB B	14 TB B	16 TB B	18 TB B	20 TB B	22 TB B
H	2 PC	4 PC	6 PC	8 PC	10 PC	11 PC	12 PC	14 PC	16 PC	18 PC	20 PC	22 PC

Figure 3: Samples and standards can be pipetted at any place in the ELISA plate. **Example** for pipetting scheme of **HUMAN IFN-γ ELISA**; standard solutions (S1 - S3) and blank (S4) as duplicate determinations; No 1 - 22 as single determinations of each human plasma sample taken from **HUMAN BLOOD STIMULATION TUBE** (NC, TB A, TB B and PC).

5. Add 50 µL / well of DET Detection Antibody Solution. Cover the plate with a microplate lid and thoroughly mix the standards / samples using a microplate shaker for 60 seconds at 500 to 1000 rpm.

   INCUBATION DURATION: 1 hour (± 5 min) at 15 - 30°C

6. Washing: Empty the wells of the plate (dump or aspirate) and wash 6 x 400 µL / well with diluted Wash Buffer.

7. Pipet 100 µL / well of CON Conjugate Solution.

   INCUBATION DURATION: 1 hour (± 5 min) at 15 - 30°C

8. Washing: Empty the wells of the plate (dump or aspirate) and wash 6 x 400 µL / well with diluted Wash Buffer.

9. Pipet 100 µL / well of TMB Substrate Solution.

   INCUBATION DURATION: 30 minutes (± 1 min) at 15 - 30°C in the dark

10. Add rapidly 50 µL / well of the STO Stop Solution onto Substrate Solution into each well to terminate the substrate reaction (no washing!).

11. Measure the absorption (OD) at 450 nm (optional reference wavelength: 620 nm). The colour is stable for at least 60 minutes.

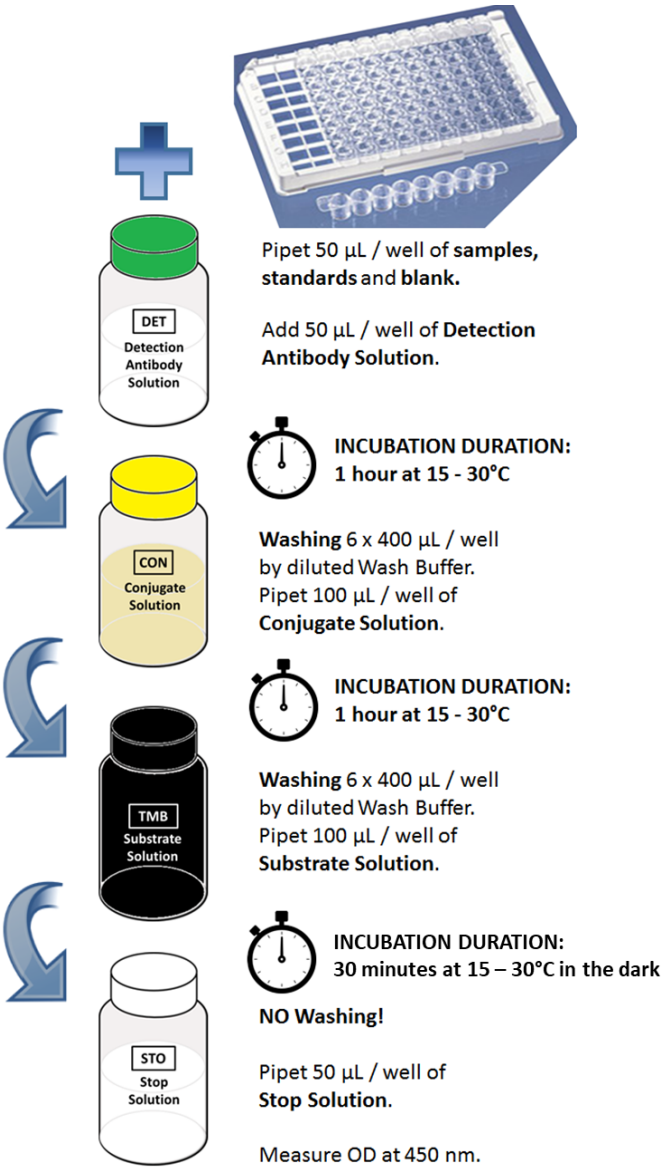

Figure 4: Schematic test procedure of using **HUMAN IFN-γ ELISA**.

Calculation and Test Interpretation

Generate a scatter diagram with logarithmic regression line. For calculation of the reference curve it is recommended to use automatic computer programmes.

Generation of standard curve

Calculate the mean OD values for the measured absorptions for every standard solution no. S1 - S3 and blank (S4).

Construct a log(e)-log(e) standard curve by plotting the log(e) of mean OD results of standard solution no. S1 - S3 on the vertical axis (y-axis) against the log(e) of concentration of cytokine (IFN-γ) on horizontal axis in IU/mL (x-axis). Omit the zero standard (Blank) from these calculations.

Calculate the line for the standard curve by regression analysis. Use the standard curve to determine the IFN-γ concentration (IU/mL) for each of the plasma samples, using the OD value of each sample.

These calculations can be performed using statistical software (e.g. Microsoft®Excel® or specific software). It is recommended that these packages should be used to calculate the regression analysis, the coefficient of variation CV (%) for the standards and the correlation coefficient (r) of the standard curve.

NOTE: For solution no. S1 - S3 and blank (S4), the difference between single values should not exceed 10 %.

In the scatter diagram with regression line, the relation between two variables is presented graphically and the regression line (the curve that fits best the plotted points) is drawn in the diagram. The equation of this curve is given in the regression window below. The resulting reference curve should be a straight line ($r > 0.98$).
Calculate the concentration of each measured sample (IU/mL) by using the log(e) equation of the reference curve:

$$\mathbf{\ln(y) = a \times \ln(x) + b}$$

Change the formula to y:

$$\mathbf{X = e^{(\ln(y) - b)/a}}$$

Example of a regression equation:

x = conc. IFN-γ [IU/mL] a = 1.1361
y = OD sample b = 1.3376

conc. IFN-γ [IU/mL] = EXP((LN(OD sample)- 1.3376)/ 1.1361)

For quantitative determination of the cytokine (IFN-γ) amount produced by antigen stimulated blood cells: Subtract the value (conc. IFN-γ [IU/mL]) calculated for the negative control NC from those observed for positive control PC and TB A and TB B (TB antigen vials). Following that, calculate 25 % of negative control value.

Interpretation of results

Note the interpretation of results carefully (Table 4)!

Table 4: Interpretation of test results.

NC Negative Control [IU/mL]	TB A - NC TB antigen A minus Negative Control [IU/mL]	TB B - NC TB antigen B minus Negative Control [IU/mL]	PC - NC Positive control minus Negative Control [IU/mL]	Result
≤ 8.00	< 0.35	< 0.35	≥ 0.50	Negative
	≥ 0.35 and < 25 % of Negative Control value	≥ 0.35 and < 25 % of Negative Control value		
	< 0.35	≥ 0.35 and < 25 % of Negative Control value		
	≥ 0.35 and < 25 % of Negative Control value	< 0.35		
	≥ 0.35 and ≥ 25 % of Negative Control value	not relevant	not relevant	Positive
	not relevant	≥ 0.35 and ≥ 25 % of Negative Control value		
	< 0.35	< 0.35	< 0.50	Indeterminate
	≥ 0.35 and < 25 % of Negative Control value	≥ 0.35 and < 25 % of Negative Control value		
	< 0.35	≥ 0.35 and < 25 % of Negative Control value		
	≥ 0.35 and < 25 % of Negative Control value	< 0.35		
> 8.00	not relevant	not relevant	not relevant	

Responses to the positive control PC can be outside the range of the microtiter plate reader. This has no impact on the test result.

Individuals with negative control NC values greater than 8 IU/mL are classified as indeterminate because a 25 % higher response of TB antigen may be outside the assay measurement range.

If the results are indeterminate, refer to section "*Troubleshooting Guide*" to identify possible causes.

Quality control of test

The **HUMAN IFN-γ ELISA** contains an internal control. The standard solutions are considered as an internal procedural control. It confirms sufficient sample volume and correct test procedure. The test result depends on the generation of a standard curve which must fulfil the following criteria to be considered as valid:

- Mean OD of S1 (standard solution 1) > 0.6
- CV (%) for S1 and S2 replicate OD values < 15%
- Variation of replicate OD values for S3 and S4 (Blank) < 0.04
- Correlation coefficient (*r*) from mean OD values of standard curve > 0.98
- Mean OD of S4 (Blank) is an internal negative procedural control and should be < 0.19

Restrictions: The reference curve should be a straight line (r > 0.98). OD values for ready-to-use standards must be within the acceptable ranges defined above (± 10 %).

If the criteria above are not fulfilled, the analysis of the test results will be affected, and the test is invalid. Insufficient sample volume or incorrect handling of the test procedure are the most likely reasons for not reaching the required QC criteria of test performance.

If the limit value of S4 (blank, see above) was exceeded, the washing procedure should be improved and/or the room temperature should be monitored during test performance (room temperature should be 15 - 30°C).

Check again the instructions of sample preparation and test procedure and repeat the test with a new microtiter strip device. If the problem persists, contact the manufacturer or your local distributor.

Limitations

Follow the instructions of the test procedure and interpretation of results carefully! Insufficient sample volume or incorrect handling of the test procedure are the most likely reasons for not reaching the required QC criteria of test performance (see section "*Quality control of test*").

IGRAs should be used as an aid in diagnosing infection with *M. tuberculosis*. A positive test result suggests that *M. tuberculosis* infection is likely; a negative result suggests that infection is unlikely. An indeterminate result indicates an uncertain likelihood of *M. tuberculosis* infection.

It is advised to consider the results from **LIOFeron®TB/LTBI** test in combination with each individual´s clinical status, results of other diagnostic tests and the background epidemiological information.

Minor confounding cases may be where infection with *M.tuberculosis* is unlikely and prevalence of some of the NTM is extraordinarily high.

The test has been developed for the detection of human IFN-γ in human plasma (Li-Heparin). For detecting IFN-γ in body fluids other than human plasma the test has not been validated and can yield incorrect results.

The blood sample should be stored at room temperature (15 - 30°C) after collection and should not be used if the sample is older than 16 hours after venipuncture. Do not freeze the blood sample. The vitality of cells can not been guaranteed after incorrect or prolonged blood storage.

Only the clear supernatant (human plasma) should be used for IFN-γ measurements. While harvesting human plasma avoid contamination by red blood cells. If necessary, separate the red blood cells from plasma by centrifugation. Avoid bacterial contamination of the samples!

The standard curve should not be extrapolated if the calculated values are non-linear. Samples showing absorbance values which exceed the highest standard concentration should be re-analyzed at higher dilution. For further analysis the human plasma samples can be diluted by IB Incubation buffer, e.g. 1:20 (50 µL human plasma in 950 µL IB Incubation buffer).

High level of circulating IFN-γ in human blood can interfere with the assay and lead to indeterminate or invalid results. Reversion and conversions can occur in IGRA tests. Various factors can affect reversion including the medications. Dynamic changes may occur which are not associated with the clinical conditions of the patients, when serially repeated. These variations, also observed in patients treated with biological agents, hardly ease the interpretation of the results. A careful clinical judgment is still required to address treatment decisions in immunocompromised patients undergoing serial IGRAs [28].

Interfering substances

Use only fresh blood samples containing anticoagulants (Li-Heparin). Do not use body fluids other than Li-Heparin human blood because other are not validated or can yield incorrect results (e.g. citrate blood inhibits assay performance).

Analytical specificity is determined by measuring potential interfering substances. Substances used for patient treatment, substances which may be ingested by the patient and substances encountered in specific specimens types are taken into account. The following substances tested in the concentrations mentioned below did not interfere test results:

Acetaminophen	20 mg/dL
Acetylsalicylic acid	20 mg/dL
Ascorbic acid	20 mg/dL
Bilirubin	60 mg/dL
Coffein	20 mg/dL
Glucose	2000 mg/dL
Haemoglobin	500 mg/dL
Lithium heparin	3 mg/dL
Tetracycline	20 mg/dL
Sodium heparin	3 mg/dL

To be on the safe side we recommend to exclude haemolysed samples from measuring because it is known that certain components of red blood cells pass into plasma and might have potential effects on the test results.

Inhomogeneity of a sample caused by an excessive amount of lipids can cause interference due to physico-chemical interactions. In addition, high concentration of lipids can potentially change the binding behavior of cytokines and thus falsify the results. Therefore, lipaemic samples may not be used.

Recent or ongoing treatment of TB may lead to incorrect results. Cytokine level in the blood may diminish rapidly after treatment. Sometimes cytokine level can be so low in patient´s blood samples that cytokines cannot even be detected in human plasma - even if an infection or disease is present.

Please note that false-positive read-outs may be caused by interference of heterophilic antibodies commonly found in human plasma from subjects with various diseases or other conditions which have not been assayed with this test.

Sodium azide and other nucleophilic substances (e.g. used as preservatives for various buffers) interfere with the activity of horseradish peroxidase. Thus, avoid the use of wash buffers or other solutions that may contain such interfering substances.

Unreliable or indeterminate results may occur due to extrapolation or deviations from the procedure.

Performance Characteristics

Measuring range of HUMAN IFN-γ ELISA

Limit of Quantitation (LoQ), limit of Detection (LoD) and Limit of Blank (LoB) of the **LIOFeron®TB/LTBI** are determined by calculating from blank values with add-on as 0.350 IU/mL (23 repetitions, different batches and operators on different days). The following values are calculated from the data measured:

LoQ = 0.2860 IU/mL LoD = 0.1360 IU/mL LoB = 0.0936 IU/mL

Comparative studies

135 samples were used to calculate concordance of **LIOFeron®TB/LTBI** to the reference method (QuantiFERON®TB-Gold Plus (Qiagen)). Concordance of **LIOFeron®TB/LTBI** to the reference method was 96.00 % for the positive group and 98.82 % for the negative group (Table 5).

Table 5: Comparative study **LIOFeron®TB/LTBI** versus QuantiFERON®TB-Gold Plus (Qiagen).

Method		QuantiFERON® TB Gold Plus*	
		positive	negative
LIOFeron® TB/LTBI	negative	2	84
	positive	48	1
Total results		50	85
Concordance		96.00 %	98.82 %

*Qiagen

Sensitivity and specificity

Analytical sensitivity, determined by measuring spiked samples with different concentration of IFN-γ within the same assay (5 repetitions each), was around 0.0625 IU/mL.

Clinical sensitivity and specificity

Whereever possible, the results of **LIOFeron®TB/LTBI** were also compared with the final diagnosis. A total of 294 samples with known diagnosis were taken into account. Out of the negative panel, 97.61 % were negative by **LIOFeron®TB/LTBI** (implies healthy individuals, HIV - and NTM patients, 251 samples). Out of the positive panel, 97.67 % were positive by **LIOFeron®TB/LTBI** (TB and LTBI, 43 samples) (Table 6).

Table 6: Summary table for results of **LIOFeron®TB/LTBI** as compared to clinical diagnosis. Abbrevations: TB = Tuberculosis, NTM = infected by other Mycobacteria, LTBI = latent TB.

Sample group	positive	negative	Total no. of samples	Concordance
positive (TB + LTBI)	42	1	43	**97.67 %**
negative (HIV, NTM + healthy)	6	245	251	**97.61 %**

Assay performance

Accuracy: For demonstration of linearity of **LIOFeron®TB/LTBI** 5 replicates each of 17 samples (plasma pool) spiked by different amounts of IFN-y are placed randomly together with the not spiked plasma pool (= zero plasma) on the ELISA plate. The results calculated (IU/mL) are plotted against the results expected. The assay has been demonstrated to be linear up to 4 IU/mL by calculating correlation coefficient r (r = 0.9953, figure 5), for concentrations up to 8.0 IU/mL the assay was approximate linear (r = 0.9824).

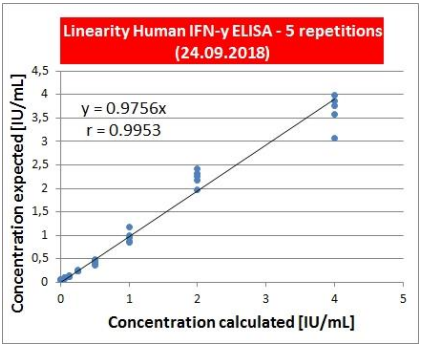

Figure 5: **LIOFeron®TB/LTBI**: demonstration of linearity of the assay between 0 and 4 IU/mL. Correlation coefficient r was 0.9953.

High dose hook effect: Human plasma was spiked by IFN-γ in concentrations up to 10000 IU/mL and spiked samples are measured by **HUMAN IFN-γ ELISA** following the instructions for use. No high dose hook effect is observed for concentrations up to 10000 IU/mL.

Precision: For estimating reproducibility of measurements, intra- and inter-assay variations as well as inter-operator variations and batch-to-batch variations were determined by measuring samples with different reactivity by **HUMAN IFN-γ ELISA**.

Inter-assay variation was determined through repeated measurements of 3 samples with different reactivity by 3 different batches on 10 different days (negative-, weak positive-, positive sample). Inter-assay imprecision (CV (%)) was 11.73% for the weak positive- and 11.9% for the positive sample. For the negative sample (= zero sample) imprecision was 27.39%. This high level of variation was expected, because of the low level of calculated IFN-γ concentration. Variation around low concentration is commonly larger than for higher concentrations**.**

Intra-assay or within run **variation** was determined through repeated measurements of samples with different reactivity (3 samples of different reactivity: negative-, weak positive-, positive sample; 20 repetitions). Intra-assay imprecision ranged from 4.35% - 10.11% (CV (%)).

Batch-to-batch variations were determined through measuring different samples with 3 different batches. Imprecision ranged from 2.52% - 19.05%. As expected, higher CV (%) above 10% was observed for the low-level samples. Around low levels imprecision is known to be higher than for higher levels. Mean CV (%) calculated for all samples was 8.37%.

For determination of **inter-operator variation** 53 samples with different reactivity were measured by 3 different operators on different days. Inter-operator variations for the OD values measured for the samples ranged from 1.465% - 31.673%. For low levels (IU/mL < 0.35) the mean CV (%) was 22.195%. For the levels above the cut off (IU/mL> 0.350 < 8.0) mean CV (%) was 12.759%.

Technical information

Indeterminate results

Test results which are analysed as indeterminate are uncommon and may relate to the immune status of the tested individuals, but may also be related to some technical factors:

- Other temperature storage conditions of whole blood as recommended (15 - 30°C)
- Insufficient shaking of **HUMAN BLOOD STIMULATION TUBES** after filling with human blood (10 x gently upside down)
- > 16 hour storage of human blood between venepuncture and incubation of blood filled **HUMAN BLOOD STIMULATION TUBES** at 37°C
- Washing procedure of **HUMAN IFN-γ ELISA**
- It is known that the rate of indeterminate result in immunocompromised patients can be higher than in other groups[26].

Clotted human plasma

Due to long-term storage of plasma samples fibrin proteins produce clots which can be accumulated by centrifugation of the plasma samples to facilitate pipetting of required plasma volumes.

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems which could occur. If the problem persists, contact the manufacturer or your local distributor (see back cover).

Table 7: **HUMAN IFN-γ ELISA** Troubleshooting Guide

Nonspecific colour development or high background	
Possible reason	**What to do**
Incomplete washing of microtiter plate	Wash the plate at least 6 times with 400 µL/well of diluted WB Wash Buffer (1x).
The expiration date of kit components has passed.	Use the kit components before expiry date. Pay attention to the expiry of reconstituted STA standard and opened MTP microtiter plate mentioned in "Stability and storage conditions of reconstituted standard and other reagents".
Incubation temperature is too high	Incubation of the ELISA should be done at room temperature (15 - 30°C).
Mixing or dilution forgotten	Ensure each step is done. Check again the instructions of sample preparation and test procedure and repeat the test with a new microtiter strip device.
Cross-contamination	Take care when pipetting the samples and solutions. If TMB Substrate Solution shows blue dye, discard the solution. Pay attention to use clean reagent reservoirs. After centrifugation of **HUMAN BLOOD STIMULATION TUBES**, do not mix the human plasma prior harvesting to avoid contamination of the sample with the gel.
Low optical density of standards	
Possible reason	**What to do**
Pipetting mistake	Pipets should be calibrated and be used according to manufacturer`s instructions.
The expiration date of kit components has passed.	Use the kit components before expiry date. Pay attention to the expiry of reconstituted STA standard and opened MTP microtiter plate mentioned in "Stability and storage conditions of reconstituted standard and other reagents".
Incubation temperature is too low	Incubation of the ELISA should be done at room temperature (15 - 30°C).
Standard dilution mistake	Ensure dilutions of the STA standard are prepared according to instructions for use.
Incubation time is too short	Incubation time of the mixture of plasma samples + DET Detection Antibody Solution is 1 hour (60 minutes). Incubation time of CON Conjugate Solution is 1 hour (60 minutes). The incubation time of TMB Substrate Solution is 30 minutes.
Reagents are too cold	All reagents should be warmed up to room temperature (15 - 30°C).
Incorrect plate reader filter is used	Plate should be read out within 60 minutes after addition of STO Stop solution at 450 nm (optional reference wavelength: 620 nm).
Non-linear standard curve and duplicate variability	
Possible reason	**What to do**
Standard dilution mistake	Ensure dilutions of the STA standard are prepared according to instructions for use.
Incomplete washing of microtiter plate	Wash the plate at least 6 times with 400 µL/well of diluted WB Wash Buffer (1x).
Insufficient mixing	Mix the STA standard stock solution during reconstitution for 10 seconds at vortexer. Be sure to mix the standard solutions S1 - S3 thoroughly prior next dilution step and addition to microtiter plate.
Incorrect continuity of pipetting technique or interruption during test procedure	Pipetting of sample and standard solutions should be performed continuously. All reagents should be prepared before starting the assay procedure.

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Symbols

IVD	For in vitro diagnostic use
CE	Compliant with IVD Directive 98/79/EG
GTIN	Global Trade Item Number
REF	Catalogue Number
LOT	Batch code
	Manufacturer
Σ	Contains sufficient amount for <n> tests
	Consumables: use by … (expiry date)

2°C 8°C	Store at 2 - 8°C (temperature limitation)
2°C 30°C	Store at 2 - 30°C (temperature limitation)
	Please consult instructions for use
	Do not reuse
	Do not use if damaged
	Protect from moisture
	Keep away from sunlight

PC	Positive control
NC	Negative control

TB A	TB antigen A
TB B	TB antigen B

MTP	Microtiter plate
STA	Recombinant human IFN-γ standard
DET	Detection Antibody Solution
CON	Conjugate Solution

TMB	Substrate Solution
STO	Stop Solution
WB	Wash Buffer, 10x concentrate
IB	Incubation Buffer

Contact Information

For more information and technical assistance, please contact

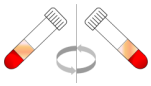
sales@lionex.de
or call + 49 (0) 531 - 260 12 66
or visit our homepage www.lionex.de

Abbreviated Test procedure

Step 1 - HUMAN BLOOD STIMULATION VIAL

Collection of whole blood from the vein: Take minimum **4.5 mL** human blood under standard laboratory conditions (aseptically, avoid haemolysis) by using **Li-Heparin** blood collection tube.

Invert the Li-Heparin blood collection tube several times upside down. Take care that the sample is homogeneous. Transfer **1 mL of the blood** sample into each **HUMAN BLOOD STIMULATION TUBE** NC, TB A and TB B and PC and **mix 10 times gently upside down**. Immediately place the **HUMAN BLOOD STIMULATION TUBES** upright in an incubator at **37°C (± 0.5 °C) for 16 - 24 hours**.

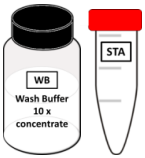


Harvest the human plasma by **centrifugation** of the tubes for **15 minutes at 2000 to 3000 RCF** (g). The red blood cells accumulate in the gel plug which separates the cells from the plasma.

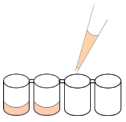
Transfer 50 µL of the clear supernatant (human plasma) directly into wells of microtiter plate and continue with step 5 of "HUMAN IFN-γ ELISA Test procedure" in instructions for use of **HUMAN IFN-γ ELISA** Kit. Optionally store the samples as indicated in "Use and storage of stimulated plasma". **Avoid contamination by red blood cells!**

Step 2 - HUMAN IFN-γ ELISA

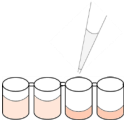
Equilibrate all kit components **to room temperature** (15 - 30°C).



Dilute WB **Wash Buffer (10 x concentrate)** to 1 x wash buffer with distilled water. **Reconstitute** STA **recombinant human IFN-γ standard** to 12.5 IU/mL stock solution by addition of indicated volume of IB Incubation Buffer and mix by vigorous shaking (e.g. vortexing for 10 seconds). **Prepare** further standard solutions **S1 - S4** (refer to section for "*Preparation of reagents*").

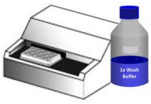


Mix every sample shortly before use. **Pipet 50 µL /well of** each **samples**, prepared STA standard solutions **S1 - S3 and** pipet IB Incubation Buffer as **blank (S4)** into the wells of MTP micro titer plate. The **standards and blank** should be measured **in duplicate**, the **samples as single determinations**.

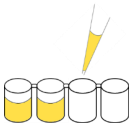


Pipet 50 µL / well of DET **Detection Antibody Solution** into the wells of MTP microtiter plate. Mix sample / standard and detection antibody thoroughly using a microplate shaker for 1 minute at 500 to 1000 rpm.

INCUBATION DURATION: **1 hour (± 5 min) at 15 - 30°C**

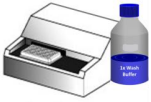


Wash the MTP microtiter plate **6 x 400 µL / well** with **diluted (1 x) Wash Buffer**.

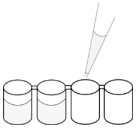


Pipet 100 µL / well of CON **Conjugate Solution** into the wells of MTP microtiter plate.

INCUBATION DURATION: **1 hour (± 5 min) at 15 - 30°C**

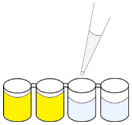


Wash the MTP microtiter plate **6 x 400 µL / well** with **diluted (1 x) Wash Buffer**.



Pipet 100 µL / well of TMB **Substrate Solution** into the wells of MTP microtiter plate.

INCUBATION DURATION: **30 minutes (± 1 min) at 15 - 30°C in the dark**



Add rapidly **50 µL / well** of the STO **Stop Solution** onto Substrate Solution into each well of MTP microtiter plate to terminate the substrate reaction (no washing!).

Measure the absorption (OD) at 450 nm (optional reference wavelength: 620 nm). The colour is stable for at least 60 minutes.

Analyse the results (**Software** or manually by following the instructions for use).

Note: please check on www.lionex.de for specific software.

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