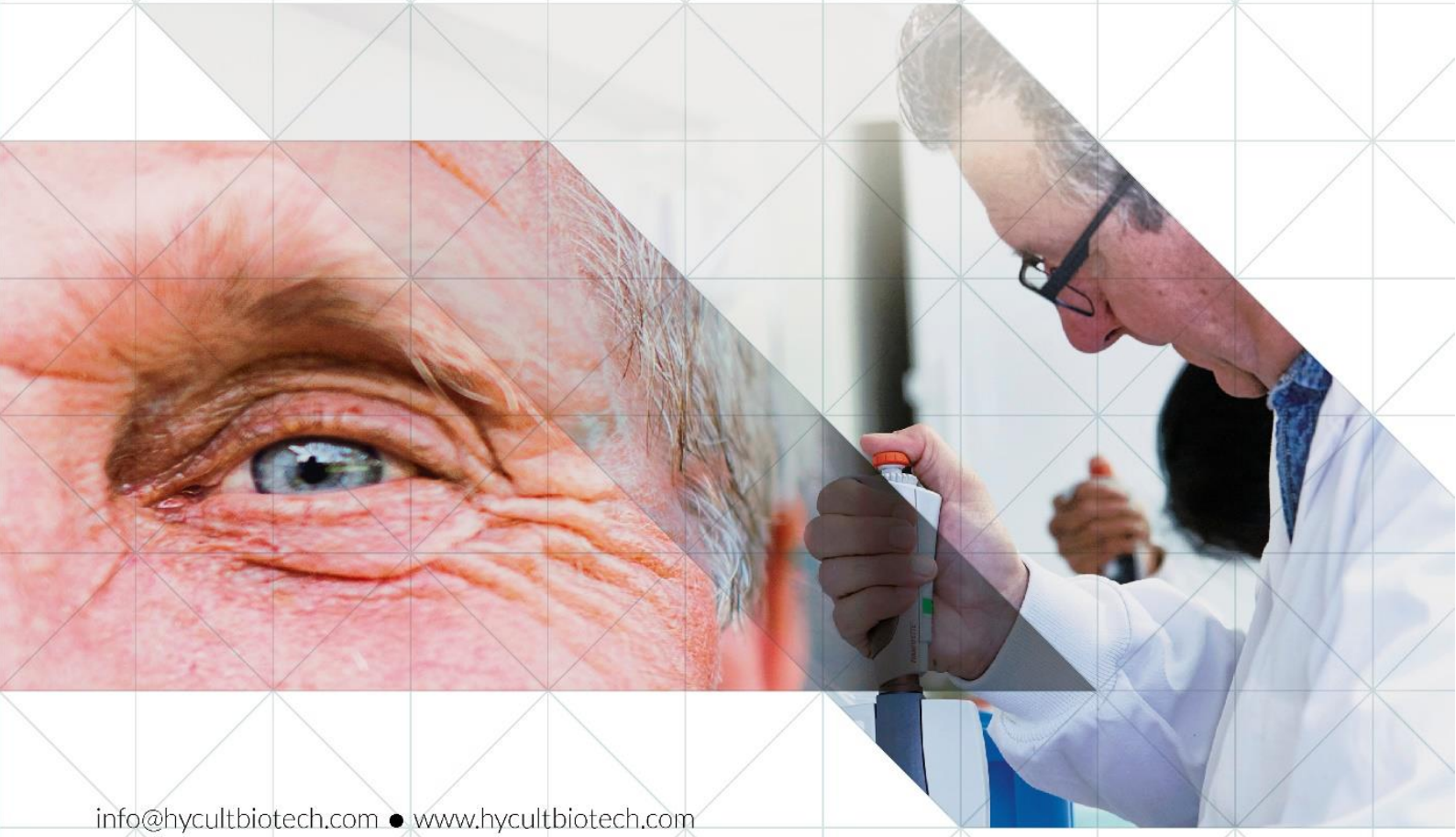
# Limulus Amebocyte Lysate Chromogenic Endpoint Assay

# HIT302

**Edition 11-18**

**ENDOTOXIN DETECTION KIT**
**PRODUCT INFORMATION & MANUAL**

Read carefully prior to starting procedures!
For use in laboratory research only
Not for clinical or diagnostic use



info@hycultbiotech.com ● www.hycultbiotech.com

Note that this user protocol is not lot-specific and is representative for the current specifications of this product. Please consult the vial label and the Certificate of Analysis for information on specific lots. Also note that shipping conditions may differ from storage conditions.

For research use only. Not for use in or on humans or animals or for diagnostics. It is the responsibility of the user to comply with all local/state and federal rules in the use of this product. Hycult Biotech is not responsible for any patent infringements that might result from the use or derivation of this product.

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## 1. INTENDED USE

The Hycult Biotech Limulus Amebocyte Lysate assay is a sensitive and specific product available to detect and measure bacterial endotoxin. The LAL assays is to be used for the *in vitro* quantitative determination of various biological fluids (including sera), devices, (air)filters and tissue culture medium. This kit is intended for laboratory research use only and is not for use in diagnostic or therapeutic procedures.

The analysis should be performed by trained laboratory professionals.

## 2. INTRODUCTION

The Hycult Biotech LAL assay is a sensitive and specific product available to detect and measure bacterial endotoxin, a fever-producing by-product of gram-negative bacteria commonly known as pyrogen. The basis of the test is that endotoxin causes an opacity and gelation in Limulus Amebocyte Lysate, LAL, this process is based on an enzymatic reaction. The simplicity and economy of the LAL test encourages the testing of various biologicals (including sera), devices, (air)filters and tissue culture medium.

Frederick Bang observed that bacteria caused intravascular coagulation in the American horseshoe crab, Limulus polyphemus. In collaboration, Levin and Bang found that the agent responsible for the clotting phenomena resided in the crab's amoebocytes, or circulating blood cells, and that pyrogen (bacterial endotoxin) triggered the turbidity and gel-forming reaction enzymatically.

The present assay makes use of the activation of an enzyme. In the presence of a colourless substrate, the enzymatic reaction will cause a yellow colour to develop upon cleavage of the chromophore, p-nitroaniline (pNA). The reaction is stopped by the addition of acetic acid and the absorbance at 405 nm is measured with a spectrophotometer. The endotoxin concentration of samples with unknown concentrations, which are run concurrently with the standards, can be determined from a standard curve.

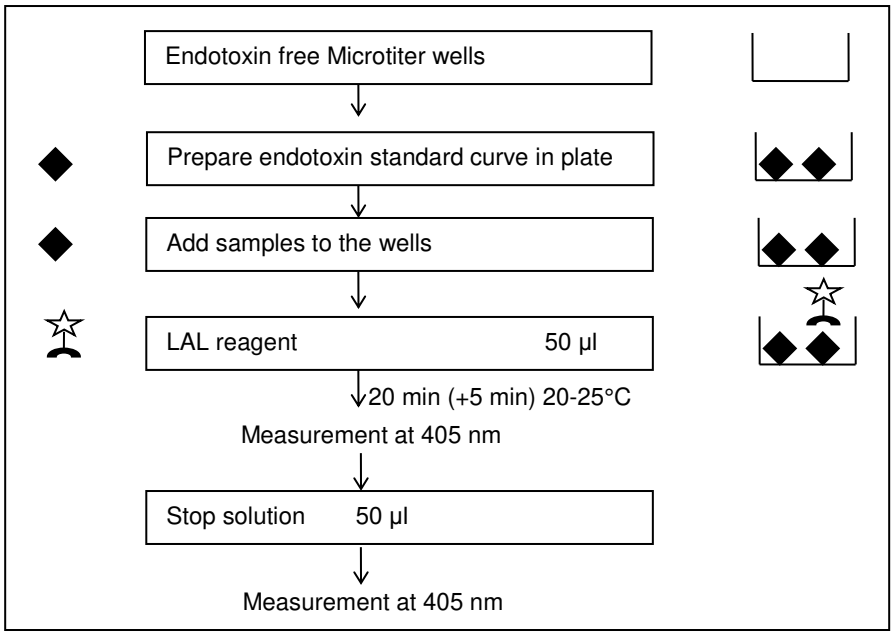
## 3. KIT FEATURES

- Working time of 20-45 minutes.
- Minimum concentration which can be measured is 0.04 EU/ml.
- Measurable concentration range of 0.04 to 10 EU/ml.
- Working volume of 50 µl/well.
- 1 vial of LAL reagent is sufficient for ~75 wells.

### Cross reactivity

Cross-reactivity for other species or proteins/peptides has not been tested.

## 4. PROTOCOL OVERVIEW




- The LAL assay is a detection and measuring method for endotoxin with a working time of 20 – 45 minutes.
- The efficient format of 3 plates allows free choice of batch size for the assay. Please notice 1 vial of LAL reagent is sufficient for ~75 wells.
- Samples and standards are incubated in microtiter wells.
- LAL reagent will bind to the endotoxin and will react by developing a yellow color.
- The enzyme reaction is stopped by the addition of acetic acid.
- The absorbance at 405nm is measured with a spectrophotometer. A standard curve is obtained by plotting the absorbance (linear) versus the corresponding concentrations of the endotoxin standards (log).
- The endotoxin concentration of samples, which are run concurrently with the standards, can be determined from the standard curve.

## 5. KIT COMPONENTS AND STORAGE INSTRUCTIONS

| Kit component | Cat # | Quantity | Color code |
|---|---|---|---|
| Standard | | 1 vial, lyophilized | Red |
| LAL reagent | | 4 vials, lyophilized | Yellow |
| Endotoxin Free Water (EFW) | DB64 | 2 vials (30 ml) | Colorless |
| Stop solution 2.5x | | 1 vial (11 ml) | Red |
| Microtiter plate | | 3 | |
| Certificate of Analysis | | 1 | |
| Manual | | 1 | |
| Data collection sheet | | 3 | |

Table 1

- Upon receipt, store individual components at 2 - 8°C. Do not freeze.
- Do not use components beyond the expiration date printed on the kit label.
- All reagents should be brought to room temperature (18-25°C) prior to use and should be stored at 2 – 8°C immediately after use.
- The E.coli standard and the LAL reagent are stable in lyophilised form until the expiry date indicated on the kit label, if stored at 2 – 8°C
- The exact concentration of the standard is indicated on the label of the vial and the certificate of analysis.
- Once reconstituted the standard is stable for 1 month if stored at 2 – 8°C.
- Once reconstituted the LAL reagent is stable for 3 hours if stored at 2 – 8°C (avoid exposure to light). If the LAL reagent has turned yellow you must use a new vial of LAL reagent for your experiment.

### Materials required but not provided

- Calibrated micropipettes and sterile disposable tips, endotoxin free.
- Vortex-type Mixer.
- Calibrated ELISA plate reader capable of measuring absorbance at 405nm.
- Depyrogenated tubes.

## 6. WARNINGS AND PRECAUTIONS

- For research use only, not for diagnostic or therapeutic use.
- This kit should only be used by qualified laboratory staff.
- Do not under any circumstances add sodium azide as preservative to any of the components.
- Do not use kit components beyond the expiration date.
- Do not mix reagents from different kits and lots. The reagents have been standardized as a unit for a given lot. Use only the reagents supplied by the manufacturer.
- The assay has been optimized for the indicated standard range. Do not change the standard range.
- Open vials carefully: vials are under vacuum.
- Do not ingest any of the kit components.
- Kit reagents can contain 2-chloroacetamide as a preservative. 2-Chloroacetamide is harmful in contact with skin and toxic if swallowed. In case of accident or if you feel unwell, seek medical advice immediately.
- The stop solution contains 50% acetic acid and can cause irritation or burns to respiratory system, skin and eyes. Direct contact with skin and eyes should be strictly avoided. If contact occurs, rinse immediately with plenty of water and seek medical advice.
- Incubation times, incubation temperature and pipetting volumes other than those specified may give erroneous results.
- Do not reuse microwells or pour reagents back into their bottles once dispensed.
- Handle all biological samples as potentially hazardous and capable of transmitting diseases.
- All materials coming in contact with specimen, test material and all contents of the kit including EFW and LAL reagent must be endotoxin-free. Glassware must be depyrogenated by validated conditions, such as 30 minutes exposure at 250°C or purchased endotoxin-free.
- A test method must be validated for each sample by demonstrating the absence of significant interference. Inhibition is usually concentration dependent, and is overcome by dilution with EFW. Common sources of inhibition include conditions that 1) interfere with the enzyme-mediated reaction, and 2) alter the dispersion of the endotoxin (positive) control. In general spiking controls should be included in all LAL protocols to detect inhibitory conditions.
- Hycult Biotech LAL assay in addition to endotoxin reacts with some β-Glucans. Materials (e.g. pipet tips) with cotton plugs are often a source of glucan and endotoxin and should be avoided.
- LAL reagent is a product from the American horseshoe crab (*Limulus Polyphemus*) and batches can therefore differ in reaction strength with endotoxin. This only has influence on the incubation times (range 20-45 minutes) to meet the specification that the 10 and 4 EU/ml should differ less than 10%. If this specification is met, the results of the experiment are good, regardless of the time of incubation.

## 7. SAMPLE HANDLING

### General

Samples should be stored preferably at –70°C or otherwise below –20°C. Avoid multiple freeze and thaw cycles which may give erroneous results. Before performing the assay, samples should be brought to room temperature (18 – 25°C) and mixed gently. Avoid foaming.

Prepare all samples (controls and test samples) prior to starting the assay procedure.

All materials or diluents coming in contact with specimen or test reagents must be endotoxin-free. Use aseptic techniques at all times. Since the LAL-endotoxin reaction is pH dependent, it is imperative that the sample-LAL mixture should yield a pH of 6.5 to 8.0. Use an endotoxin-free TRIS buffer if pH adjustment is necessary. Do not arbitrarily adjust the pH of unbuffered solutions because the Hycult Biotech LAL-assay formulation is already buffered.

When using samples which could disturb OD 405 nm measurement (for example serum/plasma) always run sample controls concurrently.

### Serum or plasma samples

In serum or plasma samples endotoxin can be measured keeping in mind to test for interference. Most reliable results will be obtained if EDTA plasma is used. After blood sampling serum or plasma should be separated by centrifugation within 20 minutes. Blood samples should be kept on ice. Use samples immediately after thawing. Serum, plasma and other biological fluids may contain endotoxin inhibiting compounds. In general these compounds can be neutralized by heating the sample at 75°C for 5 minutes.

Handle all samples and materials in contact with serum/plasma and biological fluids according to guidelines for preventing transmission of blood-borne infections.

### Devices and filters

Analyses have demonstrated that the following factors influence the detection of endotoxin present in or on filters or devices: device/filter-type, extraction solution, preservation method of device/filters. The influence of these factors can be in part estimated by spiking of endotoxin in various steps of the extraction procedure.

### Dilution procedures

We recommend to perform a serial dilution of 3 to 5 samples to determine the optimal dilution for your sample type. An example of a serial dilution is 0x (undiluted), 10x, 100x and 1000x of the samples with endotoxin free water. If the samples are undetectable, the endotoxin level is low and can be stated as <0.04 EU/ml.

### Comment regarding recommended sample dilution

The mentioned dilution for samples should be used as a guideline. The recovery of endotoxin from an undiluted sample is not 100% and may vary from sample to sample. When testing less diluted samples it is advisable to run recovery experiments to determine the influence of the matrix on the detection of endotoxin.

### Procedure for interference testing

Recovery is evaluated by interference (inhibition/enhancement) testing through spiking a sample or diluted sample with a known concentration of endotoxin and testing for spike recovery in duplicate following the assay procedure.

For interference testing select a point at or near the middle of the standard curve. The calculated mean amount of endotoxin in the spiked sample, when referenced to the standard curve, must be within 50-200% to be considered free of inhibition or enhancement. Failure to recover the spike within 50-200% indicates sample interference. Further dilute the sample in EFW until the spike is recovered consistently by the assay.

## 8. REAGENT PREPARATION

Allow all the reagents to equilibrate to room temperature (20 – 25°C) prior to use. Return to proper storage conditions immediately after use. Use only endotoxin free materials.

### LAL reagent

Prepare the LAL reagent by adding EFW to the LAL reagent vial. The exact reconstitution volume is mentioned on the Certificate of Analysis. Remove the stop of the LAL reagent vial immediately after reconstitution and cover the vial with an endotoxin free surface or the inner side of parafilm. Gently swirl until LAL dissolves into a colorless solution.

### Standard solution

Reconstitute the standard by pipetting the required amount of EFW. The exact reconstitution volume is mentioned on the Certificate of Analysis. Vortex at least 5 minutes after reconstitution. The endoxin standard has a concentration of 50 EU/ml after reconstitution.

### Stop solution

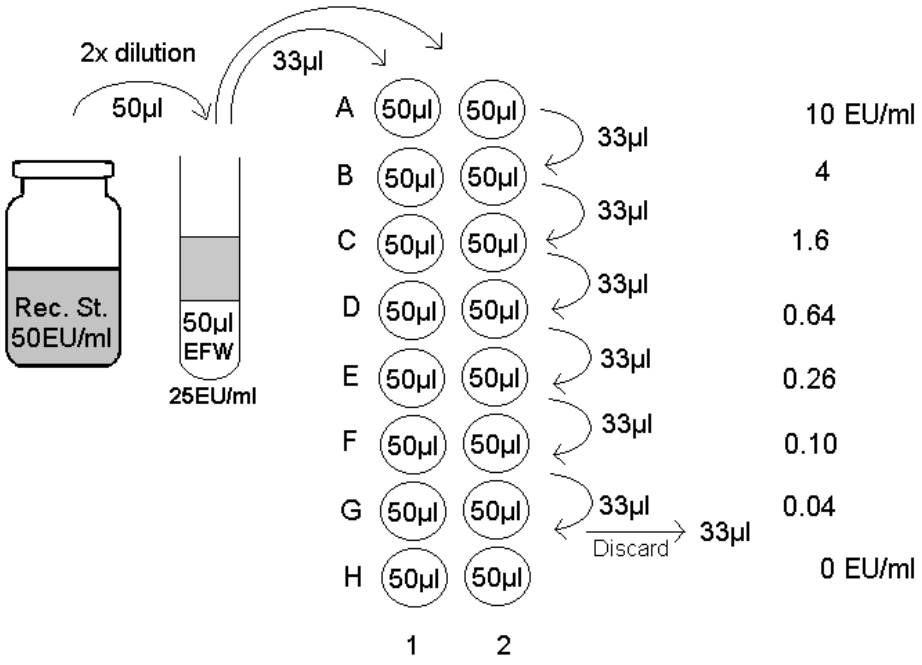
Add 10 ml of 2.5x concentrated stop solution to 15 ml distilled water, which is enough for 3 x 96 tests. In case less volume is required, prepare the desired volume of stop solution by adding 1 part of stop solution to 1.5 parts water.

Always add concentrated stop solution to water!

## 9. ELISA PROTOCOL

Bring all reagents to room temperature (20 - 25°C) before use.

1. Determine the number of test wells required to assay standards, controls and samples at least in duplicate.
2. Remove the microtiter plate from the storage bag and number the plate with a laboratory marker. To avoid condensation, do not open the bag until it has reached room temperature.
3. For standard series dilute the reconstituted standard 2 times, 50 µl standard with 50 µl EFW, to obtain a concentration of 25 EU/ml. Vortex at least 30 seconds.
4. For duplicate standard curve fill 16 wells of the plate with 50 µl EFW. Transfer 33 µl of the diluted standard to well A1 and dilute 1:1.5 further by mixing thoroughly and pipetting 33 µl over to well B1 and again to the next well and so on until well nr. G1. Discard 33 µl from well G1, well H1 will be used as control value. Repeat the standard dilution method for the A2-H2*.
5. Transfer 50 µl in duplicate from each (diluted) sample and controls to the assigned wells. Use a clean pipette tip for each transfer. When using samples which could disturb OD 405 nm measurement (for example serum/plasma) always run sample controls concurrently.
6. Add directly 50 µl/well of reconstituted LAL reagent. In case of sample controls add 50 µl EFW instead of LAL reagent.
7. Cover the plate and incubate for 20 minutes at room temperature.
8. Measure the samples at 405 nm.
9. In case the standard concentrations 10 and 4 EU/ml differ more than 10% in OD value continue incubation for an additional 5 minutes.
10. In case the standard concentrations 10 and 4 EU/ml differ less than 10% in OD value stop the reaction by adding 50 µl 1x stop solution keeping the same sequence and timing in mind as used in step 4 and 5. Gently tap the tray to mix the solutions in the wells and to eliminate any air bubbles trapped in the wells.
11. Place the tray in an ELISA plate reader and measure the absorbance at 405 nm following the instructions provided by instrument manufacturer.




* Protocol for standard dilution; Rec. St.: Reconstituted Standard, EFW: Endotoxin Free Water

## 10. INTERPRETATION OF RESULTS

- Calculate the mean absorbance for each set of duplicate standards, control and samples.
- If individual absorbance values differ by more than 15% from the corresponding mean value, the result is considered suspect and the sample should be retested.
- The mean absorbance of the zero standard should be less than 0.1 OD 405 nm. The mean absorbance of the 10 EU/ml standard should be higher than 0.6 OD 405 nm.
- Create a standard curve using computer software capable of generating a good curve fit. The mean absorbance for each standard concentration is plotted on the vertical (Y) axis versus the corresponding concentration on the horizontal (X) axis (logarithmic scale). If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.
- Samples that give a mean absorbance above the absorbance for the highest standard concentration are out of range of the assay. These samples should be retested at a higher dilution.

## 11. TECHNICAL HINTS

- User should be trained and familiar with ELISA assays and test procedure.
- If user is not familiar with the ELISA technique it is recommended that the user perform a pilot assay prior to evaluation of your samples. Perform the assay with a standard curve only following the instructions.
- Since exact conditions may vary from assay to assay, a standard curve must be established for every run. Samples should be referred to the standard curve prepared on the same plate.
- Do not mix reagents from different batches, or other reagents and strips. Remainders should not be mixed with contents of freshly opened vials.
- Each time the kit is used, fresh dilutions of standard, samples and LAL reagent should be made.
- Caps and vials are not interchangeable. Caps should be replaced on the corresponding vials.
- To avoid cross-contaminations, change pipette tips between reagent additions of each standard, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- Waste disposal should be performed according to your laboratory regulations.

**Technical support**

Do not hesitate to contact our technical support team at support@hycultbiotech.com for inquiries and technical support regarding the LAL assay.

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## 12. QUALITY CONTROL

The Certificate of Analysis included in this kit is lot specific and is to be used to verify results obtained by your laboratory. The absorption values provided on the Certificate of Analysis are to be used as a guideline only. The results obtained by your laboratory may differ.

This assay is designed to eliminate interference by soluble receptors, binding proteins, and other factors present in biological samples. Until all factors have been tested in the immunoassay, the possibility of interference cannot be excluded.

For optimal performance of this kit, it is advised to work according to good laboratory practice.

## 13. PERFORMANCE CHARACTERISTICS

### Precision and reproducibility

The intra-assay precision and reproducibility was determined with two standard vials that were applied in eight concentrations in duplicate. The intra variation had an average CV of 1.9%. The lowest observed CV value was 0.0% and the highest was 7.7%.

## 14. TROUBLESHOOTING

Warranty claims and complaints in respect of deficiencies must be logged before expiry date of the product. A written complaint containing lot number of the product and experimental data shall be sent to support@hycultbiotech.com.

General suggestions summarized below in Table 2 can be used as guideline in case of unexpected assay results.

| Low absorbance | High absorbance | Poor duplicates | All wells positive | All wells negative | Possible cause |
|---|---|---|---|---|---|
| • | • | | • | • | Kit materials or reagents are contaminated or expired |
| • | | | | | Incorrect reagents used |
| • | | • | • | | Lyophilized reagents are not properly reconstituted |
| • | • | • | • | • | Incorrect dilutions or pipetting errors |
| • | | • | | | Improper plastics used for preparation of standard and/or samples |
| • | • | | | | Improper incubation times or temperature |
| | | • | | | Especially in case of 37°C incubation: plates are not incubated uniformly |
| • | | | | | Assay performed before reagents had reached room temperature |
| • | • | • | • | • | Procedure not followed correctly |
| | | | | • | Omission of a reagent or a step |
| | | • | | | Poor mixing of samples |
| | • | | • | | Low purity of water |
| | • | • | | | Cross-contamination from other samples or positive control |
| • | • | | | | Wrong filter in the microtiter reader |
| | • | • | | | Airbubbles |
| | | • | | | Imprecise sealing of the plate after use |
| • | | | | | Wrong storage conditions |
| • | | | | | Lamp in microplate reader is not functioning optimally |

Table 2

## 15. REFERENCES


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