Optimization of the *In vitro* Pyrogen Test (IPT) Regarding Detection of Pyrogens in Air Samples

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Sammanfattning

Pyrogen kallas ämnen som framkallar feber och de kan exempelvis bestå av hela eller delar av bakterier, virus eller svamp (fungi). En metod som kallas för *in vitro* pyrogen test (IPT) har utvecklats för att detektera dessa pyrogen. Metoden bygger på att enlösning som misstänks innehålla pyrogen får komma i kontakt med blod från en människa. Efter en inkubering på mellan 4-24 timmar har blodet reagerat på eventuella pyrogen och bildat cytokiner, där mängden cytokiner är proportionell mot mängden pyrogen. De intressanta cytokinerna i den här studien var IL-1β och TNF-α, som båda är involverade i feberprocessen. Det har varit svårigheter med att standardisera metoden, mycket beroende på att det är levande celler som hela metoden bygger på, så syftet med den här studien var att förbättra *in vitro* pyrogen test. Luftprover tagna i inomhusmiljöer som misstänks innehålla pyrogen har använts i försöken att optimera varje steg i processen. De olika stegen inkluderade extraktion av filter som använts vid luftprovtagningen, inkubering med helblod och provextrakt och analys av inkuberingen med ELISA (enzyme linked immunosorbent assay). Några av de parametrar som undersöktes gällde extraktionsmedium, skaktid och skakintensitet under extraktionen, blodförhållande under helblodsinkuberingen och lämpliga cytokiner för metoden.

Studien resulterade i att en metodik, för att analysera luftprov innehållande pyrogen med *in vitro* pyrogen test, kunde tas fram.
Abstract

Pyrogens are substances that may induce fever in the human body. They can be parts of bacteria, virus or fungi and due to the reaction they may cause in the body, they are routinely looked for in the medical technology industries. A method called *in vitro* pyrogen test (IPT) has been developed to detect these pyrogens. It is based on the fever reaction in the human body and only requires blood in combination with a solution believed to contain pyrogens. If the result is positive, the production of cytokines is started. The cytokines of interest in the IPT method are those involved in the fever process and two of them are IL-1β and TNF-α, which are the cytokines used as markers of infection in this study. Since the production of cytokines is in proportion to the amount of pyrogens, the inflammation-inducing potential of the sample can be decided. Due to problems in standardizing the method, mainly because it handles with living blood cells, focus is still pointed at improving it. The aim of this study was to optimize parameters within the IPT method by analysing air samples taken in indoor surroundings believed to contain pyrogens. The different parameters included extraction of the filter from the air sampling, incubation of whole blood and sample extract and analysis of the incubation with ELISA (enzyme linked immunosorbent assay). More specific, some of the issues concerned extraction media, time and shaking intensity for the extraction, blood ratio for the whole blood incubation and cytokines suitable for the method.

A possible approach for the IPT method, when analysing air samples containing pyrogens, was reached.
# Abbreviations

<table>
<thead>
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<th>Description</th>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced Chemiluminescence</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse radish peroxidase</td>
</tr>
<tr>
<td>HSA</td>
<td>Human serum albumin</td>
</tr>
<tr>
<td><em>In vitro</em></td>
<td>Outside the organism</td>
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<tr>
<td><em>In vivo</em></td>
<td>Inside the organism</td>
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<tr>
<td>IPT</td>
<td><em>In vitro</em> pyrogen test</td>
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<tr>
<td>IL-1β</td>
<td>Interleukin-1 beta</td>
</tr>
<tr>
<td>LAL</td>
<td>Limulus Amoebocyt Lysate</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharides</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>RLU</td>
<td>Relative light units</td>
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<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-alpha</td>
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Introduction

Background
Pyrogens are a group name for substances that may induce fever in the human body. They can be parts of bacteria, viruses or fungi. For many years, the medical technology industries has had surgical equipments and implants routinely controlled for pyrogens since small amounts in contact with the blood stream can lead to severe consequences. There are two methods that have been widely used for this purpose, but each of them having drawbacks like requiring a lot of animals, being cost ineffective as well as non specific for humans. The methods are called the rabbit test (in vivo) and the Limulus Amoebocyte Lysate test (LAL) (in vitro). The rabbit test is based on the fever reaction in the rabbit after injection of the substance, whereas the LAL test is based on an immune defence reaction from a specific enzyme in the blood from horse shoe crabs, when brought in contact with a special pyrogen called endotoxins, part of a gram negative bacteria. The LAL test’s ability to only detect endotoxins is a limitation of the method and another drawback as it misses out all other sources of fever components.

A newly developed method detects all kinds of pyrogens with high sensitivity and is furthermore specific for humans since it only requires human whole blood. The method is called the in vitro pyrogen test (IPT) and is based on the human fever reaction after incubation of blood with a contaminated substance. The main constituent in this test is the release of cytokines (causes the fever reaction) and therefore some background information about these substances are necessary and the same goes for a method called ELISA (Enzyme Linked Immunosorbent Assay) that will be frequently used to analyse the outcome from the whole blood incubation, why both are listed under “Theory”.

Aim
This study was performed at Occupational and Environmental Medicine in Linköping, with the intention of optimizing the IPT method with focus on air samples taken in different indoor locations believed to contain pyrogens. The steps involved in the IPT method were air sampling, extraction of the filter, incubation with whole blood and sample extract and analysis of the formed cytokines IL-1β or TNF-α with ELISA. The task was to further investigate the different steps (except sampling) and optimize parameters with the intention to set up an efficient and robust methodology.

Formulation of problems
Teflon filters along with glass and polycarbonate filters are recommended for air sampling of endotoxins since these give a higher yield than cellulose filters [1]. In this study 25 mm Teflon filters with 0.5 µm porous diameter was placed in a filter holder connected to an air pump and placed in an environment believed to hold endotoxins. To get the contaminants at the filter to solubilize and to react with the whole blood, there are two alternatives to choose from. The filter can be applied directly to blood and NaCl [2], or extraction of the filter in pyrogen-free water followed by mixing the sample extract with the blood [3]. The reference extraction procedure for the LAL test is one hour shaking in pyrogen-free water [4], that in this study was changed to NaCl with the extraction time and shaking intensity further investigated. When LAL is the method in use, endotoxin-free water containing 0.05 %
Tween-20 (a detergent) can make the exchange of air contaminants from the filter up to three times larger during extraction [5], or even seven times as mentioned in one previous study [1]. Since the IPT method works with living blood cells, a detergent like Tween-20 cannot be used due to cell lysis. However, the fact remains that the exchange may get better, why the effect of very low concentrations of Tween-20 in the extraction media was examined. The optimal ratio between sample and blood for the whole blood incubation was also tested, as well as if centrifugation after incubation is necessary [2, 3] or not [6, 7]. According to a previous study, the experiment can be made more efficient, having time and sources of errors in consideration, if the whole blood incubation and the analysis with ELISA can be done using the same plate (the one-plate IPT method) [8]. This was further investigated. Apart from these questions, the use of blood samples from different individuals is a matter of interest. The individual production of cytokines varies [9] and so does the production from the same person from time to time, but how does it influence the interpretation of the result? The final step with ELISA was also optimized. In a series of experiments the reduction of the antibody concentration was tested in an attempt to reduce the cost per analysis. A final study analysed any difference in the IPT method using IL-1β and TNF-α.
Theory

Pyrogens

Pyrogens, also referred to as fever inducing components due to the inflammatory reaction they induce in the human body, occur more or less in all surroundings since they are constituents of organic dust that emerges from bacteria, viruses or fungi [7, 10]. When airborne pyrogens are inhaled they can cause health problems in the respiratory area and severe pulmonary diseases. High exposures are common at working places like agriculture or industries that during production releases small particles, but even households having animals can be a subject of risk [2, 3, 11]. The most frequent pyrogen are endotoxins, also called LPS (lipopolysaccharides), coming from the outer membrane of gram negative bacteria [5, 8, 11, 12]. Endotoxins can activate human cells to induce an inflammatory response even at extremely low levels, and furthermore they are very heat stable and therefore not destroyed under normal sterilizing conditions [13, 14, 15]. Endotoxins are present in tap water, air and on people’s fingers why materials are easily contaminated, which should be kept in mind during laboratory work handling these substances [14]. Within the medical technology industries it is of great importance that all devices are free from endotoxins since these may have direct contact with the bloodstream and cause severe damage, why there are routine to monitor the presence of endotoxins. There are three ways to examine whether the level of endotoxins or pyrogens have exceeded the limit of acceptance [6, 10, 16], and these methods are called the rabbit test, the Limulus Amoebocyt Lysate test (LAL test) and the \textit{in vitro} pyrogen test (IPT), also referred to as the whole blood assay. The rabbit test and the LAL test are regularly used while the IPT method is more recently developed [13], even though variants of IPT were tested already in the beginning of the 1980s [17, 18].

Description of different methods for measuring pyrogen effect

The rabbit test includes injection of the substance to be examined in a rabbit while measuring the raise in body temperature, which indicates a pyrogenic reaction. Various pyrogens can be detected but possible drawbacks from this \textit{in vivo} test may be that the result only represents the rabbit and it is expensive and time-consuming. A fact that contributes to the search and use of alternative methods is the amount of animals required to analyse samples in routine work that rarely contains pyrogens [7, 8, 13]. Previous studies have shown that rabbits and humans react differently towards certain pyrogens (although LPS had similar effects between the two species), indicating that some questions will remain unanswered about the relevance to humans [19]. An alternative method is the LAL test which is an \textit{in vitro} test using a lysate taken from the blood of horseshoe crabs. This lysate is of interest since it coagulates if endotoxins are present. Limitations from this assay are that it only detects endotoxins, although with a very high sensitivity, and the information is neither this time specific for humans. Along with these disadvantages, the population of horseshoe crabs is getting smaller, even if most of them are put back into the water after the procedure. The remaining test and focus of this report is called the \textit{in vitro} pyrogen test or the whole blood assay, and combines the benefits from the previous methods by being very sensitive to a range of pyrogens, not just the endotoxins [7, 8]. The test is performed \textit{in vitro} and is surprisingly easy to carry out despite the complex reaction it is based on, since it only requires blood samples from healthy donors. It is performed in a 96-well plate, where blood and pyrogens are mixed and incubated for some hours (for how long depends on the time to produce the cytokine chosen as a marker
of infection) in 37° C in air containing 5 % CO₂. The cells are allowed to interact with the pyrogens and start producing cytokines, in other words fever inducing agents [6].

The IPT method is the best test to examine the effects of pyrogens in humans as it focuses on the fever reaction in human blood (the formation of cytokines), and furthermore it can detect all sorts of pyrogens on a range of materials (solution, powder or solid material) [20]. The experiments are followed by analysis with ELISA to measure the level of the cytokine [7].

Cytokines

To begin with, the main cytokine analysed here is called IL-1β, even though analyses with another cytokine called TNF-α also was made. These cytokines are the first to be released from the macrophages when the body is exposed to pyrogenic agents, why they can be used as markers of infection (see Figure 1). To defense the body against an infection, one important thing they do is to raise the body temperature, which is an effective host defense [21]. The more inflammation-inducing substances there are, the more cytokines are produced so the reaction can be used to quantify the inflammation-inducing potential of the sample [3], which is useful to conclude whether a special sample (air samples, injectable drugs or samples from some kind of surface) is toxic or not.

![Diagram of cytokines](image)

**Figure 1.** A schematic view of the actions of various cytokines released from activated macrophages. The cytokines of interest in this study are IL-1β and TNF-α [21].

ELISA with chemiluminescent detection

The ELISA assay is used to detect or quantify antibodies or antigens.

Plates used in ELISA are coated with a capture antibody (or an antigen if the target molecule is an antibody) that binds to the surface of the wells. The capture antibody is designed to later on bind to the molecule of interest. Since the capture antibody is unable to cover the whole surface in each well, a blocking agent (for example BSA, bovine serum albumin or HSA, human serum albumin) has to be added to saturate all plastic binding sites in the wells. The plates are often made to be suitable for a range of different molecules. A MaxiSorp plate from
*Nunc* (used in this study) has high binding capacity for proteins and molecules with both hydrophilic and hydrophobic regions, like antibodies. Due to the many molecules suitable for the surface, it is important to avoid unspecific binding. Other types of plates have a surface more specified to certain molecules [22].

At this point the ELISA plate is ready for the sample which is added together with a biotinylated detection antibody that will bind in to the target molecule (see Figure 2). When streptavidin-HRP (horse radish peroxidase) is added, streptavidin will form a stable complex with all biotin molecules while the unbound tail HRP are free to react with the final reagent ECL+ (Enhanced Chemiluminescence plus) and produce a chemiluminescent signal (light) that is in proportion to the amount of the target molecule [22, 23].

In this study, the target molecule represents the cytokines IL-1β or TNF-α. During the incubation with samples (or standards), the IL-1β or TNF-α that might have been generated during the whole blood incubation for 18 or 6 hours depending on the cytokine, will bind to the capture antibodies (anti IL-1β or anti TNF-α) in the wells.

*Figure 2.* A scheme that illustrates the ELISA analysis. In this study 384-well plates were used and instead of the chromogene TMB that is shown in the figure the chemiluminescence substrate ECL+ was used. Each well represents one sample and undergoes the same steps during the analysis [24].
Materials and Methods

For all experiments, fresh human whole blood drawn into heparinised tubes (BD Vacutainer®, Plymouth, U.K) have been used and the ELISA set with matched antibodies and standards was from Diaclone, Besancon, France. All materials were as clean as possible, meaning everything that could be autoclaved was and the rest was washed with 70 % ethanol and left to dry in 50°C. LPS standard solutions made in every test where somewhere between 0 and 1000 ng/ml.

Air sampling

Samplings were made using air pumps (CASELLA CEL, Bedford, U.K) with a flow rate of 2 litres/min and the Teflon filters used had a pore size of 0.5 µm. Samples were collected in a garbage room of 7.5 m³ and in an exposure chamber with a volume of approximately 4 m³ in which 2-5 weeks old household garbage had been stored under closed ventilation. These were chosen since a parallel project aimed to study if the smell could be caused by pyrogens. To obtain a positive control, samples were also collected under a rug (that occasionally was shook) in a home that had a cat. This procedure was described in an earlier study [1].

Extraction

The parameters that were studied were the effect of low concentrations of Tween-20 in the extraction media (0.9 % NaCl) and the effect of time and shaking intensity for the exchange of pyrogens.

When LAL is performed 0.05 % of Tween-20 can be used which increases the exchange from the filter. The drawback when the same procedure is used in the IPT method is that the blood cells get disturbed and cell lysis may be an issue if the amount is too high. To find a level of Tween-20 low enough to be tolerated by the blood cells, three columns (with eight wells each) in the 96-well plate were used. One just contained NaCl (NaCl 0.9 % B.Braun, Melsungen, Germany) and Tween-20 (Sigma-Aldrich, St. Louis, USA ) in different concentrations (0.0005, 0.005, 0.05 and 0.5 %), the other two LPS (Lipoplycosaccharides from Salmonella enterica serotype minnesota, Sigma-Aldrich, St. Louis, USA) of 0.01 and 0.001 ng/ml respectively, together with the different concentrations of Tween-20. The LPS standards were previously known to stimulate the production of IL-1β, why they were chosen. The total volume was 250 µl, which in the first column contained 200 µl of Tween-20 solution, 25 µl of NaCl and 25 µl of blood, while the rest had 200 µl of Tween-20 solution, 25 µl of LPS solution and 25 µl of blood.

When the effect of Tween-20 was analysed the filter was cut in two pieces, leaving one to get extracted in 4 ml of NaCl and the other in 4 ml of NaCl containing 0.005 % Tween-20. The extraction procedure took place on a shaker with 400 rpm for one hour followed by centrifugation for 5 minutes at 3000 rpm. LPS standard solutions in both NaCl and NaCl with 0.005 % Tween-20 were made. All solutions were transferred to a 384-well plate (MaxiSorp plate No 460372, Nunc, N.Y, USA) or a 96-well plate (polypropylene plate No 267245, Nunc A/S, Roskilde, Denmark) depending on one-plate experiment or two-plate experiment (see detailed information under Whole blood incubation - one plate vs. two plates). For one-plate experiment 64 µl of extract and 16 µl of whole blood were transferred to coated and blocked wells of a 384-well plate. For two-plate experiment 240 µl of extract and 60 µl of
whole blood were transferred to a 96-well plate and allowed to incubate for 18 hours. The supernatants were then transferred to a coated and blocked 384-well plate.

For the evaluation of extraction time and shaking intensity, half a filter was added to 4 ml of NaCl in an autoclaved vial and extracted for one hour on a horizontal shaker (400 rpm) and the other half for ten minutes on a smaller horizontal shaker (2000 rpm), followed by centrifugation (3000 rpm for 5 minutes). The test was performed using either the one-plate or two-plate methodology with 20% of whole blood (as described above).

**Whole blood incubation**

Blood was collected from voluntary donors and drawn into heparinised tubes. Since the activity of the blood cells had to be as similar as possible to how they behave in the body, it was used within four hours. Within whole blood incubation, five parameters were subjects of interests; the blood content, different blood donors, any difference in the result between the cytokines IL-1β and TNF-α, centrifugation of the cell mixture after incubation or not and the use of one plate or two separate plates for the whole blood incubation and ELISA.

In the first test analysing the difference in information between blood contents, four were selected with a range covering standard amount to a very small amount: 20, 10, 5 and 2.5%. The whole blood incubation was performed in a 96-well plate (two-plate IPT). Standard solutions of LPS and NaCl were made. The total volume in the wells was set to 250 µl, leaving LPS to fill out the remaining volume after blood had been added (note: when small volumes of blood are added by an electronic micropipette it is easier to dip the pipette in the LPS solution, why blood can be added last). After gently stirring by drawing the pipette slowly back and forth, the plate was allowed to incubate for about 18 hours (to reach a maximum level of IL-1β) in a cell culture chamber (37°C in an atmosphere having 5% CO₂, LabRum Klimat Ab, Stockholm, Sweden).

Further analyses were done with 40, 30, 20 and 10% of whole blood, analysing the different reactions towards LPS standard solutions. The procedure was done using both the one-plate and two-plate methodology.

Further experiments analysed different blood volumes’ reactions towards an extract solution from a contaminated filter. 40, 30 and 20% of whole blood were used and the incubation was performed in a 96-well plate (two-plate IPT).

Four different blood donors were used to analyse their reactions towards LPS standard solutions. A volume of 225 µl of LPS solution and 25 µl (10%) of blood were added to each well in a 96-well plate.

Further tests were made with the incubation performed in cluster tubes made of polypropylene (Corning Incorporated, New York, USA) containing 20% of blood and LPS standard solutions.

A comparison between the cytokines IL-1β and TNF-α, in regard to examine their ability to act as markers of infection, was done as well. Blood samples from one or several donors and LPS standard solutions or sample extract were added to a 96-well plate or to cluster tubes (20% of whole blood). The procedure was the same as before, except for the incubation time. TNF-α is formed within 4-6 hours instead of within 18-24 hours as for IL-1β, why the
incubation was stopped after 5 hours and the supernatant placed in the freezer until the incubation for IL-1β was ready. The supernatants where then transferred to separate ELISA plates, coated with either anti IL-1β or anti TNF-α.

The air samples that were used in the experiment above, when comparing the cytokines IL-1β and TNF-α (positive control, garbage room and exposure chamber) were analysed by the LAL test as well, to compare with the results from the IPT method. The filters were cut in half, leaving one to get analysed by the IPT method (as described above) and the other by the LAL test.

To decide whether centrifugation was necessary or not, LPS standard solutions were made and 225 µl of each were added to a 96-well plate followed by adding 25 µl of blood (10 %). The incubation was performed as previously described. Normal procedure at this point was to transfer all liquid (blood and standard solution) in the wells to small cluster tubes and centrifuge for 5-10 minutes (2500 rpm), which was done to one column (eight wells). The other column, with the exact same composition, had 40 µl of the supernatant directly transferred to the ELISA plate using an eight-channel pipette.

The experiment was repeated, but instead of having the blood incubation in a 96-well plate it was performed directly in the cluster tubes (pre-washed with 70 % ethanol and left to dry in a heating cabinet in 50°C) with the advantage of not needing any extra transferring step. The total volume was set to 350 µl, whereof 20 % was blood and the rest LPS standard solution. Incubation was performed as previously described, followed by centrifugation of eight cluster tubes (to be compared with the eight wells in the 96-well plate) while transferring the supernatants of the other eight cluster tubes, with the exact same composition, directly to the ELISA plate.

For the reporting on one plate versus two plates, whole blood incubation and ELISA will be discussed together since they are performed in combination.

For the two-plate experiment, air sample extracts or LPS standard solutions of different concentrations were gently mixed with the blood in 96-well plates and allowed to incubate over night in a cell culture chamber. The ELISA plate was treated separately with coating antibodies and incubated over night in the fridge. The following morning, after the ELISA plate being blocked, were the samples from the incubation added to the plate to quantify the amount of cytokines (see Figure 3). The one-plate experiment required a 384-well plate coated with anti IL-1β antibodies (capture antibodies) before the adding of sample extracts or LPS standard solutions and blood. This plate is usually utilized when ELISA is run, explaining why the wells are already coated during the whole blood incubation for 18 hours. Under the whole blood incubation, no matter which method are used, it is important that every step and solution are as endotoxin-free as possible since the blood cells can get activated by extremely small doses.
The first test with one-plate was a repetition of the study described by Poole et al [8], except for some small details (IL-1β was used as a marker instead of IL-6 and TNF-α, saturation buffer was changed to BSA instead of the more expensive HSA (human serum albumin), the volumes of sample, blood and detection antibody were a bit different and a 384-well plate was used instead of a 96-well plate). Despite the rearrangements, the result was assumed to be interpreted likewise. To blocked wells, 50 µl of LPS standard solutions were added, as well as 20 µl of blood and 10 µl of detection antibody (provided in the IL-1β set and further diluted before use 100 and 500 times, respectively, in PBS (phosphate-buffered saline) containing 1 % of BSA). The incubation was performed in a cell culture chamber as previously described.

The experiment was repeated once more but this time the amount of reagents followed the laboratory manual and detection antibody was not added until after the whole blood incubation. To each well 64 µl of LPS standard solution of different concentrations was added together with 16 µl of blood (20 %).

The previous experiment was further changed when exclusion of saturation buffer (still not adding the detection antibody until after the whole blood incubation) was investigated. To each well 64 µl of LPS standard solution was added along with 16 µl of blood.

A variant of the last experiment was performed using diluted serum instead of 5 % BSA in PBS as saturation buffer. It was made by taking 1 ml of the supernatant from the blood after it had been centrifuged and dilute it in 3 ml of NaCl. The serum that was used was taken from the same person whose blood was used in the whole blood incubation. To a 384-well plate, still containing 40 µl of coating buffer (anti IL-1β), 40 µl of diluted serum was added and allowed to incubate for one hour in room temperature while slowly stirring (200 rpm). After thorough washing, LPS standard solutions and blood were added as before.

**ELISA**

The analysis with ELISA follows a description provided in the IL-1β set. Due to the use of a 384-well plate instead of a 96-well plate and detection by chemiluminescence instead of absorbance, less reagents were needed to achieve the same result. In short, the wells to be
used were coated with 40 µl of capture antibody diluted 200 times in 0.05 M potassium carbonate buffer with pH 9.6 and allowed to bind in to the surface over night in the refrigerator. The next day, the wells were washed twice with 100 µl of a washing solution containing PBS (phosphate-buffered saline) with 0.05 % of Tween-20, followed by adding 100 µl of 5 % BSA in PBS (blocking solution) to react for two hours while gently stirring (200 rpm) in room temperature. The washing procedure with the washing solution was repeated and 40 µl of sample (supernatant from extraction- or standard solution and blood from the whole blood incubation) was added in replicates of two along with 40 µl of detection antibody diluted 100 times in PBS with 1 % BSA. The diluting factor was, however, experimented with. The plate with the mixture was stirred like before for three hours, letting the cytokines bind in to the capture antibodies. The wells were washed three times with the washing solution before the adding of 40 µl of streptavidin-HRP (diluted 230 times in PBS with 1 % BSA and 0.1 % Tween-20). The plate was stirred like previously described for 20 minutes, followed by washing three times with washing solution and two times with water. 40 µl of the final reagent ECL+ (diluted 1+7 in milli-Q-water) (GE Healthcare, Buckinghamshire, U.K) was added and placed dark for 15 minutes before the chemiluminescence was measured using a chemiluminescence plate reader (Lumistar, BMG labtechnologies, Offenburg, Germany). The description for the ELISA procedure (see Table 1) was made for two-plate IPT and was therefore adjusted under one-plate IPT.

Table 1. The steps in ELISA, which are the same for both IL-1β and TNF-α.

<table>
<thead>
<tr>
<th>Steps in the ELISA analysis</th>
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<tbody>
<tr>
<td>Coating</td>
<td>18 h (over night)</td>
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<tr>
<td>Blocking</td>
<td>2 h</td>
</tr>
<tr>
<td>Sample- and Detection antibody incubation</td>
<td>3 h</td>
</tr>
<tr>
<td>Streptavidin-HRP incubation</td>
<td>20 min</td>
</tr>
<tr>
<td>ECL+</td>
<td>15 min</td>
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</table>

There is not much to change that could contribute to an improvement in the analysis with ELISA, except for lowering the concentration of the detection antibody and thereby reduce the cost of the analysis, and by combining the ELISA plate with the plate for whole blood incubation.

The experiments aimed to study how much detection antibody that was needed were performed in the beginning of the study and the lowest, but still sufficient, concentration was then used throughout the rest of the study. Standard solutions of IL-1β ranging from 0-1000 pg/ml were made. The lowest positive control was set to a concentration of 15.6 pg/ml. To a 384-well plate coated with anti IL-1β and saturated with 5 % BSA in PBS, 40 µl of the standard solutions were added followed by adding 40 µl of detection antibodies in concentrations of 100, 50 and 25 % of what is recommended by the company producing the ELISA set. Streptavidin-HRP and ECL+ was added as described above.
Statistics
For the calculations of differences between groups, students t-test two-sample equal variance by Microsoft® Office Excel has been used. All graphs and quantifications of LPS equivalents in unknown samples were done using GraphPad Prism 4 version 4.03.
Result

The results from the experiments will be presented in the same order as they were presented in “Materials and methods”.

Extraction

Since Tween-20 increases the exchange from the filter during extraction but at the same time have a negative effect on the blood cells, experiments were made to find a level as low not to affect the cells but high enough to benefit the extraction. First the concentration of Tween-20 tolerated by the blood cells was examined, which had to be decided before the possible effect on the extraction could be further evaluated. When analysing the results from the different Tween-20 concentrations the following parameters were controlled:

- Tween-20 was not allowed to induce a pyrogenic reaction, why all concentrations ranging from 0 to 0.5 % of Tween-20 in some wells only contained NaCl instead of LPS. If Tween-20 did not induce a pyrogenic reaction, all concentrations should have the same RLU signal (relative light unit, a measure of chemiluminescence and index of the formation of cytokines) as 0 % of Tween-20 in NaCl (the background noise).

- In the wells containing LPS, there should be a similar cytokine formation until the amount of Tween-20 reaches the level of cell lysis. The RLU signals are smaller due to a decreased production of IL-1β (see Figure 4).

Given that Tween-20 appeared to be free from pyrogens and an amount up to 0.005 % did not affect the blood cells, the conclusion was that 0.005 % of Tween-20 could be used in the extraction experiments with filters used for air sampling.

![Figure 4. The effect of the presence of Tween-20 in the extraction media on the formation of IL-1β in the whole blood incubation. RLU (relative light units), that are in proportion to generated IL-1β, was used as an index of IL-1β. The first attempts to extract filters in both NaCl and NaCl with 0.005 % of Tween-20 did not lead to the expected results. As already investigated, that amount of Tween-20 should have](image-url)
the same effect on the blood cells as pure NaCl but when the results from the extraction were compared, the Tween-20 solutions had much higher RLU signals. Although it was the intention of the study, the enhanced signals did not depend on an increased yield but on contamination in some step. A parameter that differed from the test deciding the amount of Tween-20 was that the Tween-20 solutions were prepared in polypropylene tubes instead of in polystyrene tubes. It seemed like Tween-20, when in contact with the surface of the polypropylene tubes, generated pyrogens. As a result, the tubes were switched to polystyrene tubes and similar results as previously were received. However, due to similar results when using either of the extraction solutions (NaCl or NaCl with 0.005 % Tween-20), it was finally established that Tween-20 did not increase the pyrogen exchange from the filter.

The extraction time and shaking intensity were evaluated, with the filter extracted in NaCl for one hour on a shaker with 400 rpm, or for ten minutes on a shaker with 2000 rpm. The result showed no difference between the extraction procedures, why it was decided that the extraction could be made equally efficient only requiring 10 minutes of vigorous shaking.

**Whole blood incubation**

The original blood volume when two plates where used (two-plate IPT) was 50 µl in a total volume of 250 µl (20 %) (Charles River’s patented assay, Endosafe®-IPT). In this study, blood sample ratios of 2.5 to 40 % were used in order to find an ultimate relation. The observation was that the sensitivity of the assay increased when lower percentage of the whole blood was used in the incubation (see Figure 5). The finding was that 20 % of blood was the most suitable amount for this method.

![Figure 5. The formation of IL-1β for different blood volumes after stimulation of LPS. The dotted line shows the dose-response curve using 20 % of blood.](image)

The results from the studies with different blood donors and different cytokines (IL-1β or TNF-α) are presented under *Detection of pyrogens in ambient air samples* further below.

Centrifugation has the disadvantage of requiring one extra transferring step (from the incubation plate to cluster tubes) which increases the sources of errors. When comparing the
IL-1β generated by LPS and blood that was centrifuged after the whole blood incubation with the generated IL-1β by LPS and blood that was transferred directly to the ELISA plate, no clear difference could be seen. When the incubation was performed in a 96-well plate the centrifugation step was therefore excluded in all future experiments. However, if cluster tubes were used for the incubation it was considered just as well to centrifuge since no transferring step was required.

Due to a high response even from a very small amount of LPS (0.00001 ng/ml) that normally do not induce a cell activity, small changes were done to eliminate the problem (autoclaving or washing with 70 % ethanol of almost all materials, more blood since more blood had turned out to react first towards a stronger concentration of LPS, new LPS standard solution as well as repeating the same procedure several times in case of a diluting problem) but without improvement. The use of cluster tubes for the whole blood incubation where used to once again see if any difference between centrifugation or not was shown, and with these tubes a transferring step was excluded. The result indicated like before that it is not crucial to centrifuge and what was more, the standard curve acted as anticipated with no reaction towards the smallest amounts of LPS (see Figure 6). Further analyses to evaluate the tubes role in receiving an acceptable standard curve showed the same result, why the incubation from this time forth was performed in cluster tubes instead of in a 96-well plate.

![Figure 6](image)

Figure 6. Formation of TNF-α expressed as RLU after treatment of 20 % whole blood with LPS between 0.0001 and 100 ng/ml during five hours. The dose-response curve shows mean ± SD of two replicates.
Normally, the IPT method is performed in two steps; 1) incubation of whole blood and sample in polypropylene tubes (cluster tubes) or in the wells of a polypropylene plate and 2) analysis of the cytokine in a separate ELISA plate. However, the question asked was which to choose. Repeating the method described by Poole et al [8], although not using the same cytokines, did not lead to the same result. According to his study, capture antibody, blocking solution and detection antibody were included under the whole blood incubation. Use of two plates has none of these solutions in contact with blood cells under the whole blood incubation. As capture antibody was impossible to rule out due to the claim of catching cytokines, the two other solutions were tested. To block any binding site left, the previous study used HSA while only BSA was available in this study. After tests using BSA versus non-BSA the result indicated that BSA had an effect on the blood cells. Further studies were done evaluating the detection antibody’s role as a source to why the experiment failed, coming to the conclusion that it also might have contributed to the high level of endotoxins. As a result, new tests were done without saturation buffer with BSA and detection antibody not added until the analysis with ELISA took place, which gave an improved result, although it had a tendency to have a higher background noise than when using two plates. Due to that, the dose-response curve got a shorter interval, which is not suitable when quantifying unknown samples.

As a final attempt to obtain acceptable results, diluted serum was used as a blocking agent with the intention to reduce the signal so it would behave more like the signal when two plates were used. The assumption was that the proteins in the serum, among others HSA, would bind in to the well. Because of no success in reaching lower levels, no further studies were made to improve the method.

**ELISA**

The result from the experiment trying different amounts of detection antibody indicated that half of the original dose (original dose = diluted 100 times, recommended by Diaclone, Besancon, France) can be used since it could detect a concentration of IL-1β at 15.6 pg/ml, as the original amount. The use of 25% of the original amount had the RLU signal from 15.6 pg/ml not significantly separated from the background noise. However, all three concentrations of detection antibody showed linearity between 15.6 pg/ml and 1000 pg/ml (see Figure 7).
Detection of pyrogens in ambient air samples

Air samples collected in three different locations (a house holding a cat, a garbage room and an exposure chamber with household garbage collected within five weeks) were analysed to evaluate the efficiency of the IPT method, using the new parameters that were established above (extraction in NaCl for ten minutes, 20% of whole blood, no centrifugation after incubation and two-plate IPT). The IPT method’s ability to detect pyrogens, when using different blood donors and different cytokines (IL-1β or TNF-α), are presented here.

The experiments with different blood donors and their cells ability to react on pyrogens and generate IL-1β or TNF-α were performed in a 96-well plate or in cluster tubes. Since parallel experiments for IL-1β and TNF-α were performed any difference in response between those could also be seen. According to Table 2 the results for the positive control with TNF-α are consistent between the blood donors (0.313 and 0.226), compared to IL-1β which have different LPS equivalents between the blood donors in the positive control (0.032 and 0.609). Noticeable is also, that the LPS equivalents between the cytokines are diverse. Moreover, in the IPT test for TNF-α it appears to be pyrogens in the garbage room not detected in the same test using IL-1β (even though the amounts are close to the non-detectable level). A previous test with one blood donor, using samples from the same locations, indicated pyrogens in both the garbage room and the exposure chamber for TNF-α while nothing was seen for IL-1β. The tendency leans towards TNF-α being the better cytokine of these two for this kind of test, regarding its ability to detect small amounts of pyrogens as well as providing stable results.

The same samples as used above were run in the recognised LAL test to see how well they corresponded, even if the methods do not take the same parameters in consideration. The LAL test counts endotoxins per m³ air through the pump, while the IPT method in this study refers the samples to the standard curve where the standard solutions have LPS concentrations in ng/ml. The IPT method thereby counts all the endotoxins stuck on the filter and does not consider the time the air pump was on. Therefore, the LAL value can not be translated to LPS equivalents, but rather give a direction to if the samples are positive or not (see Table 2). According to Figure 8, both the IPT method and the LAL test detected endotoxins in sample 29 (the positive control), even if the LAL test this time was five times more sensitive.
Table 2. Levels of endotoxins in three samples measured with both the IPT method and the recognised LAL test. For the IPT method, two blood donors and two markers of infection (IL-1β and TNF-α) were used to see any difference in the result between them. The values in the LAL test give an indication to if endotoxins were detected, which they then should be in the IPT method as well. The levels that were below the blank sample (NaCl and blood) were marked as non-detectable = nd.

<table>
<thead>
<tr>
<th>Sample</th>
<th>IPT (LPS equivalents, ng/ml)</th>
<th>LAL (ng LPS/m³ air)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blood donor 1</td>
<td>Blood donor 2</td>
</tr>
<tr>
<td>Blank</td>
<td>0</td>
<td>0.0002</td>
</tr>
<tr>
<td>Positive control¹</td>
<td>0.032</td>
<td>0.609</td>
</tr>
<tr>
<td>Garbage room²</td>
<td>nd.</td>
<td>nd.</td>
</tr>
<tr>
<td>Exposure chamber³</td>
<td>nd.</td>
<td>nd.</td>
</tr>
</tbody>
</table>

¹ The positive control represents a sample taken in a home holding a cat. The pump was placed under a carpet for about 3 hours (air flow 2 litres/minute) while being whipped on at several occasions.
² Air sampling in a garbage room of size 7.5 m³.
³ Air sampling in an exposure chamber of 4 m³ with no ventilation, containing household garbage collected within five weeks.

Figure 8. The same sample analysed with both the IPT method and the LAL test. The LAL test could analyse the sample first when diluted five times. Sample 29 represents the positive control.
Discussion
The different parts will be discussed separately, leaving some to be more deeply discussed than others depending on the problems during the study.

Extraction
The idea of adding Tween-20 in the extraction solution came from studies working with LAL tests, where Tween-20 added to pyrogen-free water increased the yield of endotoxins extracted from the filter. After finding a concentration that was tolerated by the blood cells, the experiment started, giving a result that was not expected. Even though the Tween-20 concentration previously had proven to be alright, the signal indicated contamination in some step. Replacing the Tween-20 solution with a new one did not help, whereas the introduction of other tubes did give acceptable RLU signals. This indicated that either did the polypropylene tubes contain pyrogens or did Tween-20 solubilize endotoxins from the plastic surface.

When the problem was solved, with the introduction of new tubes, the second question was if the presence of Tween-20 in the extraction solution increased the yield of endotoxins from the filter. After receiving a filter exposed to air, the experiments started. Despite problems with the LPS standard solutions (the blood reacted intensively towards a small amount of LPS that was not supposed to induce an activity and the RLU signal did not increase when the LPS concentration got higher) the conclusion was made that Tween-20 did not increase the yield. The fact is, however, that several studies on endotoxins with the LAL method have shown that endotoxins are better dissolved in the extraction media if Tween-20 is present. It is possible that Tween-20 enhances the ability of pyrogen-free water to act as an extraction medium just as much as the salt molecules in NaCl, or that the low concentration of Tween-20 (0.005 %) was too low to have an effect on the filter.

Whole blood incubation
The sensitivity of the assay increased when the content of blood in the incubation mixture was reduced. It can perhaps be explained by more LPS per blood cell or that few blood cells lack the protective environment caused by more blood. The blood behaves best in its natural environment, why it can be too sensitive if diluted too much. To receive an acceptable standard curve with no reaction towards the smallest amounts of LPS (~ 0.00001-0.001 ng/ml), a higher content of blood was assumed to reduce the problem, but it remained. Probable causes could be contamination in the 96-well plate (which was one out of few materials not autoclaved or washed with 70 % ethanol) or that the formation of IL-1β somehow was unstable (see why further below). The decision to use 20 % of blood was chosen because it acted most suitable for unknown samples. The claim was no reaction at the low levels and a gradient with a plateau around LPS stimulation of 1-10 ng/ml. Although the claim of no reaction towards the smallest amounts was not met (see Figure 5), it was decided that 20 % was the most suitable blood content, since the reaction was assumed to be caused by contaminated material or instability of the cytokine (IL-1β).

During the whole period of laboratory work, different blood samples have been used that can be compared. They show that blood from different people, as well as blood from the same person taken at separated times, react differently towards pyrogens. The human body’s
physical condition varies on a daily basis due to infections, but causes no problem since standard solutions always are present in every experiment. This leads to that possible blood samples already containing cytokines, or in other ways not acting normal, can be ruled out. The drawback is nevertheless that these blood samples get discovered first when the experiment is finished and two days of work are wasted. In larger studies, all blood samples are controlled for acute infections and sensitivity before use which can be made possible thanks to cryopreservation [26, 27, 28]. Cryopreservation allows the blood cells to be frozen in aliquots, with minimum loss of the blood’s activity. Since fresh human whole blood has to be used within four hours, there is no time to test the blood’s suitability for the IPT method. Due to the many problems in this study to receive an acceptable standard curve, my opinion is that cryopreservation is necessary to have time to control the blood samples before the experiment starts.

As discovered when studying the variation in LPS equivalents between blood donors, the receiving of an acceptable standard curve is crucial. After repeating several experiments, most of them with IL-1β but also with TNF-α, the results indicated that TNF-α was better suited for this assay than IL-1β. For some reason the standard curves with TNF-α were better with a broader range, allowing LPS between 0.01 and 1 ng/ml to be determined (see Figure 6). Most of the standard curves using IL-1β failed due to a reaction towards even the smallest amounts of LPS. A probable cause could be the time difference from when they are released from the macrophages. Since TNF-α is released before IL-1β (after 4-6 hours), not so many factors are involved as they may be after 18-24 hours. Degrading may be a problem, as well as increased activity of the blood cells after such a long time outside the body. When changing the incubation to be performed in prewashed cluster tubes instead, the results for IL-1β got better, but were still not as stable and robust from time to time as the results with TNF-α.

LAL test was performed on the same samples as in the IPT method to see if both methods were able to detect endotoxins in a similar way. It was found that the sample representing the positive control contained endotoxins, using either of the methods (see Table 2). Moreover, in Figure 8, it is shown that the LAL test this time detected the positive control with a greater sensitivity than the IPT method. However, it confirms that the IPT method did detect endotoxins that the LAL test detected, which was the result we hoped for. It supports the fact that the IPT method can be used as a complement to the LAL test.

In this study, it was not possible to use a one-plate version of the IPT method. Poole et al [8] have previously done that using TNF-α and IL-6 as markers (instead of IL-1β) and with pyrogen-free antibody solutions. The reason to why we, in this study, could not use an one-plate methodology was probably due to the need of an entirely endotoxin-free environment that we could not receive. Studies to adapt the one-plate test to our set of antibodies were carried out, evaluating the role of each substance (capture antibody, blocking solution and detection antibody) in the contamination. A test not adding the detection antibody until after the whole blood incubation did not reduce the background signal, which indicated contamination in either the capture antibody and/or the blocking solution. Further tests with blocked wells versus non-blocked wells (with the detection antibody not added until after the whole blood incubation), indicated that either was the BSA solution contaminated with endotoxins, or did the blood cells react on it because of being non-human. Consequently, to be able to consider a future use for one-plate IPT, BSA was ruled out and the detection antibody not introduced until the ELISA procedure started.
To reach a low background, an effective blocking agent is an important factor [22], especially since a MaxiSorp plate has high binding capacity towards a range of molecules. Therefore, BSA could not be excluded without being replaced by something else. The hypothesis was that the HSA and other human serum proteins in the blood under the incubation could act as a blocking agent instead of BSA and bind in to all binding sites on the surface not covered by coating antibodies. Also, if detection antibody was added after the whole blood incubation, the need of an endotoxin-free solution was not crucial since there were no living blood cells left. When this experiment failed, a last try using diluted serum as blocking agent was made. The assumption was like the previous hypothesis that all the proteins in human blood would bind in to the surface. The fact was that both substitutes to BSA did decrease the background signal but not as much as expected. Probable causes could be that there were not enough proteins in the blood to block every possible binding site, leaving room for the detection antibodies to bind in and give a high background, or that the solution with capture antibodies contained endotoxins. Capture antibodies are necessary for IL-1β to bind in and were therefore impossible to exclude, why the investigation of its separate role as a contaminator had to be left out. There is also a possibility that longer incubation times increase the risk of an unspecific activation of the cells, considering the 18 hours of whole blood incubation that is necessary to produce IL-1β compared to four hours for TNF-α (which Poole et al used in his study) [8]. The idea of one-plate IPT is nevertheless a step towards making the in vitro pyrogen test easier to perform with no transferring steps and saving of incubation time. Therefore, with endotoxin-free reagents provided in the ELISA set, additional studies could better evaluate its capacity.

**ELISA**

The decision to use half the recommended dose of the detection antibody was chosen since it could detect IL-1β as good as the original amount. A lower concentration was not chosen, due to the need of an acceptable detection level in unknown samples.
Conclusion

Everything considered, the procedure for *in vitro* pyrogen test when analysing air samples could be as follows:

**Extraction**
- Extract the filter in 0.9 % NaCl for ten minutes under vigorous shaking (2000 rpm) in room temperature.

**Whole blood incubation**
- Add the extract solution to washed cluster tubes (70 % ethanol) or to an uncontaminated 96-well plate.
- Add 20 % of whole blood and place the cluster tubes or the 96-well plate in a cell culture chamber with 37˚C to incubate.
- Do not centrifuge the solutions after the incubation if performed in a 96-well plate. If the incubation was performed in cluster tubes, centrifugation requires no transferring steps and can be done without any loss of solutions.
- Use TNF-α instead of IL-1β and stop the incubation after 4-6 hours.

**ELISA**
- Add the supernatants from the samples, no matter if centrifuged or not, to the ELISA plate together with the detection antibody of half the recommended concentration (diluted 200 times).

**Future perspectives**

Recently, a breakthrough within the IPT method was presented with the introduction of the use of cryopreserved blood [26, 27, 28]. Before that, the chance of replacing the rabbit test and the LAL test was smaller due to the difficulty to standardize the method. Future analyses should concentrate on facilitating the method, for example further evaluate cryopreservation. This procedure will eliminate the inter-individual variation of the IPT method, as well as eliminating the risk of using blood not suitable due to infection or sensitivity. The possibility of using the same plate (one-plate IPT) for the whole blood incubation and the ELISA analysis should also be looked into more closely, since it may simplify the method considerably.
References


23. GE Healthcare, Amersham ECL plus western blotting detection reagents.


