2 MATERIAL AND METHODS

Preliminary investigations were aimed at choosing the best method for detecting endotoxin. This chapter includes section 2.1 for main investigations and 2.2 for preliminary investigations.

2.1 MAIN INVESTIGATIONS

It is described what was used for main investigations.

2.1.1 DETERMINATION OF ENDOTOXIN

To measure endotoxin the Kinetic-QCL-Assay Kits of „BioWhittaker“ Walkersville, MD, USA were used. All kits had the same lot number: 7L 1520. Same lot was chosen to exclude differences in behavior of *Limulus* Amebocyte Lysate reagent when comparing different methods of extraction. It was also previously reported that high differences were found when the same sample was measured with different lots [3, 28].

2.1.1.1 REAGENTS OF THE ASSAY KIT

The assay kit of BioWhittaker contains the following items.

2.1.1.1.1 KINETIC-QCL REAGENT

Eight vials containing a co-lyophilized mixture of lysate prepared from the circulating amebocytes of the horseshoe crab, *Limulus polyphemus*, and chromogenic substrate.
The reagent was reconstituted immediately before use with 2.6 ml of LAL Reagent Water (per vial) and swirled gently to avoid foaming.

2.1.1.1.2 LPS (ESCHERICHIA COLI 055:B5 ENDOTOXIN)

Vials containing 50-200 EU lyophilized endotoxin. The reconstitution volume of the vial is stated on the certificate of analysis, included with each kit, and is calculated to yield a solution containing 50 EU/ml. They were reconstituted with the specified volume of the solvent for the samples (LAL Reagent Water or Tween 20 solution or TAP buffer) and shaken vigorously for at least 5 minutes at high speed on a vortex mixer. Prior to use, the solution was warmed to room temperature and vigorously vortexed for 5 minutes. This is important because the endotoxin tends to attach to glass [82].

2.1.1.1.3 LAL REAGENT WATER

Vials containing 30 ml of LAL Reagent Water with an endotoxin activity of < 0.005 EU/ml stated by the manufacturer. This water was used to rehydrate the Kinetic-QCL reagent.

2.1.1.2 ASSAY PROCEDURE

Vortexing of all samples in every dilution step for at least 30 seconds is very important in preventing endotoxin from adhering to the glass an thus effecting the results [83]. Additionally it prevents inhomogeneous distribution of endotoxin [82].

2.1.1.2.1 STANDARD CURVE

A standard curve is needed on every microtiter plate to determine endotoxin content of the unknown samples. It was created by preparing solutions of 50 EU/ml, 5 EU/ml, 0.5 EU/ml, 0.05 EU/ml and 0.005 EU/ml (Table 2-1) like described below.

TABLE 2-1 GENERATING THE STANDARD CURVE

	Activity	Volume of Solvent: LAL REAGENT WATER / TWEEN 20 SOLUTION / TAP BUFFER	Volume of endotoxin solution added to Solvent
1. Standard	50 EU/ml	Reconstitution	-
2. Standard	5 EU/ml	0.9 ml	0.1 ml of 50 EU/ml
3. Standard	0.5 EU/ml	0.9 ml	0.1 ml of 5 EU/ml
4. Standard	0.05 EU/ml	0.9 ml	0.1 ml of 0.5 EU/ml
5. Standard	0.005 EU/ml	0.9 ml	0.1 ml of 0.05 EU/ml

Reconstituting the endotoxin standard vial created the standard of 50 EU/ml. The 5 EU/ml solution was made by adding 0.1 ml of the highest standard to 0.9 ml of solvent (LAL Reagent Water or Tween 20 solution or TAP buffer). The 0.5 EU/ml solution were received by adding 0.1 ml of the 5 EU/ml standard into 0.9 ml of solvent and so on.

When measuring the assay in the reader the software will determine a standard curve. The mean onset times are calculated and plotted as shown below (Figure 2-1). The lowest standard of 0.005 EU/ml needs the longest time (about 1 hour) to reach the threshold of 0.2 OD. The highest standard with an activity of 50 EU/ml only needs about 0.1 hour.

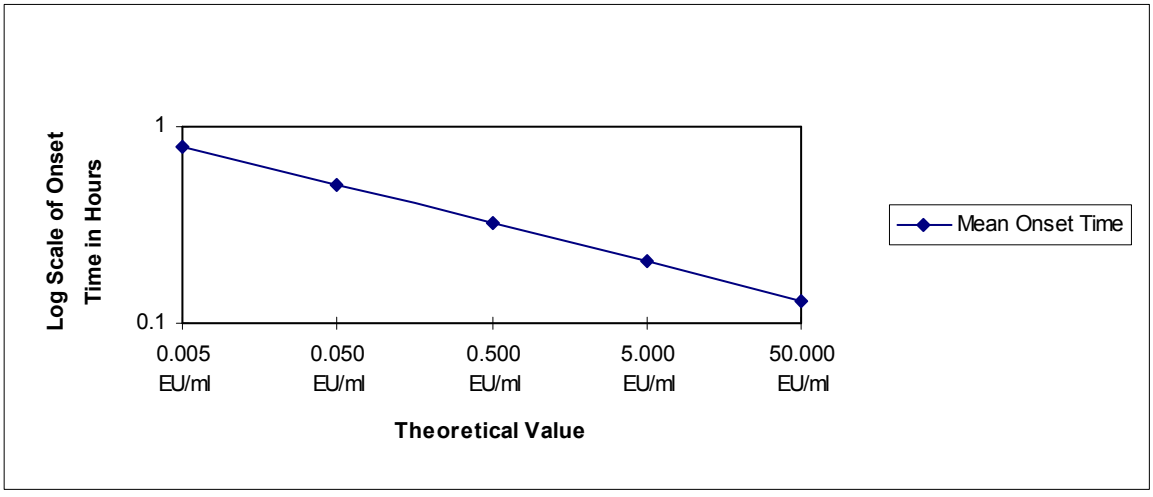

FIGURE 2-1 PLOTTING OF A STANDARD CURVE

2.1.1.2.2 DETECTION OF INHIBITION OR ENHANCEMENT

The LAL-test can be affected by many factors. Inhibition or enhancement has to be avoided and the spiking procedure was used for its detection.

Every sample was spiked with 5 EU (positive product control (PPC)) in duplicate. This activity was chosen because of its center position in a logarithmic standard curve. In consideration of its place in the standard curve 0.5 EU/ml could be taken and should if the expected activity is lower than 1 EU/ml [82]. However, because of the higher costs that would arise if the samples have to be measured again when the activity of the sample is < 1 EU/ml it were only 5 EU spikes used for the PPC. The activity of 5 EU/ml was obtained by adding 10 µl of the 50 EU/ml standard into the specified wells. A sample volume of 100 µl was added to the spike to gain an additional spike-activity of 4,5 EU/ml. When the assay has run the mean activity of duplicate sample measurements has been subtracted from mean activity of the duplicate spiked samples (PPC). The endotoxin spike recovery needed to be 4.5 EU/ml ± 50% (≥ 2.25 EU/ml ≤ 6.75 EU/ml) to be valid. (See 2.1.5.2)

2.1.1.2.3 MICROPLATE

Microplates that were used have 96 flat bottom wells (12 columns by 8 rows) (Co. Becton Dickinson Labware), stated endotoxin activity of < 0.5 EU/ml per well. Pyrogenic contamination was never found. First the 10 µl spike was placed on the microplate into the appropriate wells. All glass tubes with specimen were vortexed for at least 1 minute prior to be placed on the microplate or to be diluted. Then 100 µl of the sample were added to the spike and placed into the wells designated for it. The microplate was set into the reader of Co. Dynatex Elektronik™ and shaken for 5 seconds in a specific modus in order to mix spike and sample. Wells reserved to the standard curve were filled with 100 µl of appropriate standard solution. One blank was also measured in duplicate to ensure that the solvent was not contaminated with endotoxin.
When all specimens are loaded into the plate, see Figure 2-2, the plate was incubated at 37°C for 10 minutes on an incubating plate. Then 100 µl of Limulus Amebocyte Lysate reagent were added by a repetitive pipette to ensure that the reaction starts at the same time in the standards and in the samples. Immediately the microplate was placed into the preincubated reader at 37°C. The microplate was shaken for 5 seconds and measured every 10 seconds for 100 minutes. The microplate reader of Co. Dynatex Elektronik™ ascertained spectrophotometrically the optical density at 405 nm.

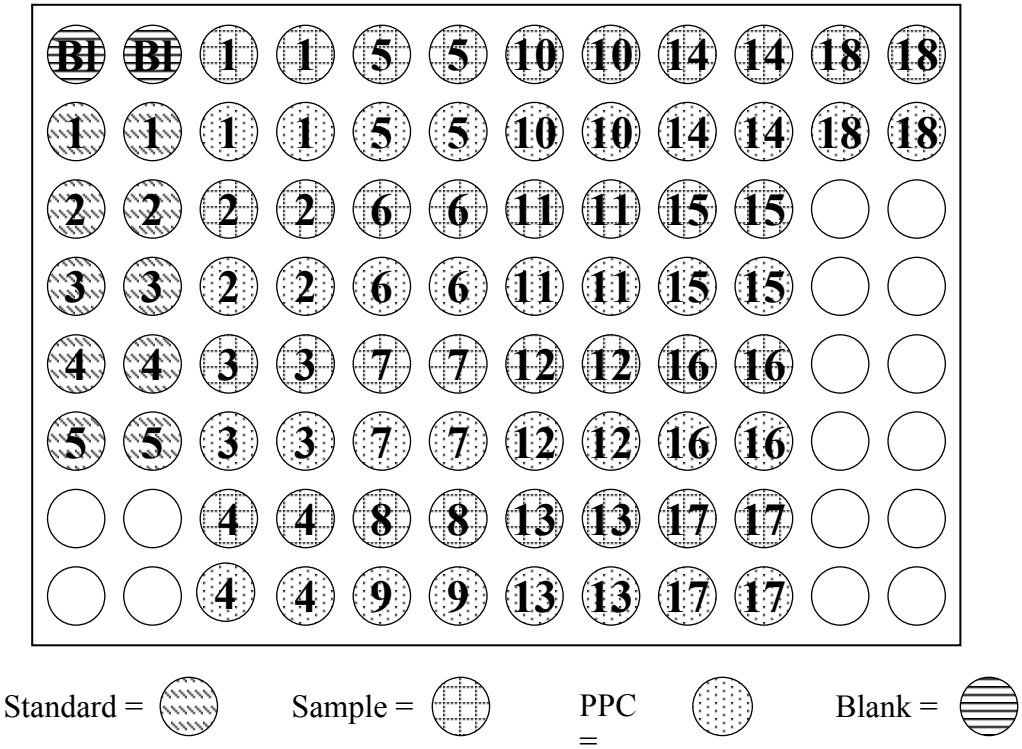

FIGURE 2-2 THE MICROPLATE SETTING

2.1.2 FILTER MATERIAL

The three most common materials of filters for heating, ventilation and air-conditioning systems (HVAC) in Germany were used.

The filters used for these investigations were made of synthetic fiber, glass fiber and cellulose fiber materials. Tested material came from a storage facility of Co. Delbag, Berlin. It was delivered to the manufacturer in large rolls. Three pieces each 1.5m x 0.5m from the deep layers of the rolls were taken to prevent larger contamination with dust. The material was brought to the laboratory and punched with stamp machine ("Knie-Hebel-Stanze" Co. IMETEX) into circular 3217 mm^2 pieces with a diameter of 64 mm. For practical use these round pieces were bisected (1608.5 mm^2) prior to use. Filter materials are not stable at high temperatures (180°C) (2.2.4.3) that are used to render things free of endotoxin. The basic activities of endotoxin for 10 randomly taken pieces were determined for each filter type (3.2.2).

2.1.3 SOLUTIONS

Solutions used in the study are:

2.1.3.1 WATER

All solutions are made with bottled (1 liter) distilled pyrogen free (< 0.25 EU/ml) water („Aqua ad injectablia“) Ph. Eur. from Delta-Pharma GmbH Pfullingen (Lot No.: 22709B). The water was tested in each assay as a blank. Only activities less than 0.005 EU/ml were found.

2.1.3.2 SOLUTIONS CONTAINING ENDOTOXIN FOR CONTAMINATION OF HVAC FILTERS

Five solutions containing different kinds or activities of endotoxin were produced as shown in Table 2-2 and sections 2.1.3.2.1 - 2.1.3.2.4.

TABLE 2-2 USED ENDOTOXIN

No.	Kind of	Activity	Solvent	Origin
1.	LPS (*E. coli* 0:55:B5)	4,000 EU/ml	H_2O*	Co. Sigma (Phenol water extracted)
2.	LPS (*E. coli* 0:55:B5)	400 EU/ml	H_2O*	Co. Sigma (Phenol water extracted)
3.	*Pseudomonas aeruginosa* (whole bacteria)	≈ 400 EU/ml	H_2O*	ATCC No.: 15442
4.	*Escherichia coli* (whole bacteria)	≈ 4,000 EU/ml	H_2O*	Isolated from HVAC filter
5.	Dust	≈ 400 EU/ml	H_2O*	HVAC (filter + duct)

*) Distilled and nonpyrogenic (< 0.005 EU/ml)

2.1.3.2.1 LPS SOLUTION

To obtain LPS-Solution the Standard Catalogue No. 210-SE of Sigma™ Chemical Company, St. Louis, MO, USA was used. It contains 10,000 - 20,000 EU per vial (*Escherichia coli* 0:55:B5 lipopolysaccharide). It was reconstituted with appropriate activity of endotoxin free water as stated on label to obtain a stock solution of 4,000 EU/ml. When reconstituted the vial was vortexed over a 30 minutes period.

2.1.3.2.2 SUSPENSION CONTAINING *PSEUDOMONAS AERUGINOSA*

Pseudomonas aeruginosa suspension was obtained by cultivating a ATTC bacteria No.: 15442. It was cultivated on tryptic soy agar up to an amount of 1.9 10^9 cfu (≅ 377,580 EU/ml). Then it was suspended in nonpyrogenic water and autoclaved at 121°C with 2,000 hPa for 20 minutes and in addition it was diluted 1:60 with nonpyrogenic water to get a final activity of 6,293 EU/ml.

2.1.3.2.3 SUSPENSION CONTAINING ESCHERICHIA COLI

Cultivating bacteria, which were found on a filter in a heating, ventilation and air-conditioning system (HVAC) in the zoo of Berlin obtained *Escherichia coli* solution. It was the only viable bacteria, which was found on a filter during an investigation done by the institute of hygiene over a period of several years. The germ was cultivated on tryptic soy agar up to an amount of 8.9 10^9 cfu (≅ 381,250 EU/ml). Then it was suspended in nonpyrogenic water. The suspension was autoclaved at 121°C with 2,000 hPa for 20 minutes and in addition it was diluted 1:1,000 with nonpyrogenic water to get a final activity of 381.25 EU/ml.

2.1.3.2.4 SUSPENSION CONTAINING DUST

Dust suspension was obtained by taking dust from used filters and out of the air conditioning ducts. The dust was sifted through a test sieve (Serial No. 464494, Standard: DIN/ISO 3310-1) with a mesh width of 63 micron to separate the dust from other particle matter like parts of the filter that would block the pipette tips. 5.40g of dust were taken and suspended into 100 ml of nonpyrogenic water. The suspension was placed into an autoclave at 121°C with 2,000 hPa for 20 minutes and in addition it was diluted 1:1 with nonpyrogenic water to get a final activity of 509.0 EU/ml.

2.1.3.3 EXTRACTION MEDIA

Two different extraction media were used to remove the endotoxin from the filter (Table 2-3).

TABLE 2-3 USED SOLVENTS

	Components	Solvent
TAP buffer	0.05 M di-Potassiumhydrogenphosphat (K_2HPO_4) and 0.01% Triethylamine ($C_6H_{15}N$), pH 7.5	H_2O*
Tween 20	0.005% Tween 20 ($CH_3(CH_2)_{10}$)	H_2O*

*) Distilled and nonpyrogenic (< 0.005 EU/ml)

2.1.3.3.1 TAP BUFFER

Triethylamin phosphate (TAP) buffer first described by Milton [72] was chosen because of its ability to avoid dilution dependent inhibitory (DDI) and resistance to interference by pH and ionic strength [23, 72]. It was prepared by using 0.05 M di-Potassiumhydrogenphosphat waterfree (K_2HPO_4) CH.-No.: B407209 634 of Co. Merck and 0.01% Triethylamine buffersubstance ($C_6H_{15}N$) for biochemical use. Lot No.: L294817 643 of Co. Merck at a pH 7.5.

2.1.3.3.2 TWEEN 20 SOLUTION

Tween 20 solution was taken because of its ability to avoid micellar formations of LPS [21] and was described as a potent source to detect higher activities of endotoxin [21, 30]. Tween 20 (Polyoxyethylen[20]-sorbitan-monolaurat) is a non-ionic detergent with a critical miscellaneous concentration (CMC) of 5.9 10^{-5} mol/l [84]. At a concentration of 0.0059% and higher the Tween 20 molecules form micelles and are a possible source of influencing the Limulus Amebocyte Lysate assay. The used concentration of 0.005% Tween 20 yields the highest endotoxin activity with smallest effect to the LAL-Test like shown in preliminary investigations (2.2.3).

Tween 20 for molecular biology use was received from Co. Merk with Lot No.: S1998580642

2.1.4 PHYSICAL CONTAMINATION AND EXTRACTION PROCEDURE

Round pieces of filter (3217 mm^2) were punched from each material and bisected with a pair of scissors. The parts were each placed into a sterile petri dish. Five samples of the same filter media were inoculated with 0.5 ml of the same contamination fluid. In addition, the same amount of contamination fluid was added to an extraction glass tube without a filter (positive test tube (PTT)) (see Figure 2-3). The filters and the PTT were placed under a laminar flow bench for about 20 hours for desiccation. The dry filter pieces were each placed in an extraction glass tube. Extraction fluid (100ml) was added to all six glass tubes. The tubes were shaken at 200 rpm for 1 hour on a shaker or placed in an ultrasonic bath with 35 kHz for 45 minutes (Table 2-4). Since preliminary investigations indicated that the highest endotoxin activity is recovered in this manner. After extraction about 3 ml form every extraction solution was poured into a corresponding new glass tube to prevent readsorbtion of endotoxin to the filter. These samples (100µl) were placed on a microplate. The LPS needed for the standard curve was reconstituted and diluted with the same solution as used for filter extraction.

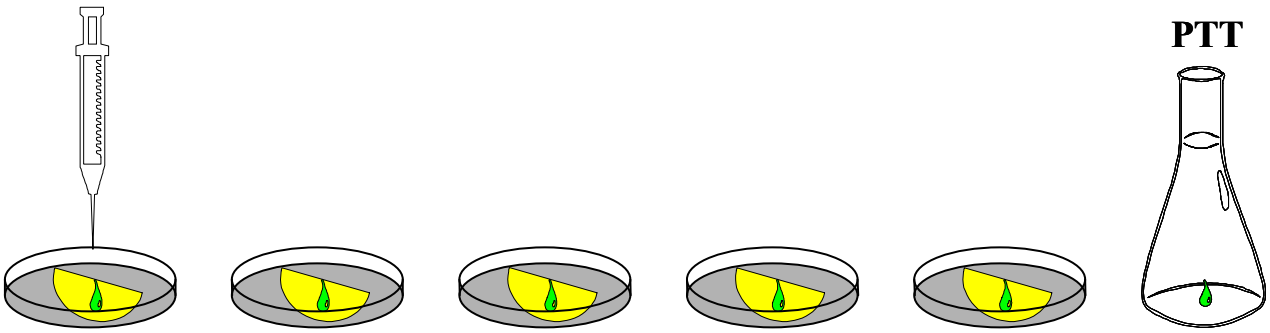

FIGURE 2-3 INOCULATION OF FILTER PIECES AND PTT

TABLE 2-4 EXTRACTION METHOD

Extraction Method	Time	Device	Intensity	Company
Shaking	1 hour	shaker	200 rpm	Centromat™ of Co. Braun
Sonication	45 minutes	ultrasonic bath	35 kHz	Sonorex™ RK 510 of Co. Bandelin

2.1.5 HANDLING OF EXTRACTION

Table 2-5 explains how the samples for main investigations were examined. Five punches of each filter material were inoculated with a specific type (in line three) and activity (line four) of endotoxin. Presented in line two there were for reference three test tubes (positive test tube (PTT)) (one for each filter material) with endotoxin contamination but without a filter piece. All combinations were shaken or sonicated in Tween 20 or TAP (line 5 and 6).

TABLE 2-5 SETTING OF MAIN INVESTIGATION

Kind and # of Filters	5 pieces of SYNTHETIC FIBER filter and 5 pieces of GLASS FIBER filter and 5 pieces of CELLULOSE FIBER filter																	
# of Reference Vials	3 extraction vials without filter (PTT)																	
Type of Contamination	LPS from Co. Sigma								*Pseudomonas aeruginosa* ATCC				*Escherichia coli* (wild type from HVAC)				Dust from HVAC	
Activity of Endotoxin	0.5 ml with 200 EU				0.5 ml with 2,000 EU				0.5 ml with 2,000 EU				0.5 ml with 200 EU				0.5 ml with 200 EU	
Extraction Solution	100 ml of 0.005 % Tween 20		100 ml of TAP															
Physical Extraction Method	sonication for 45 minutes	shaking for 1 hour at 200 rpm																
Number of Vials	# 18	# 18																
Total Number of Vials	# 360																	

2.1.5.1 TIMETABLE OF MAIN INVESTIGATIONS

The total setting of samples was divided into 20 smaller parts. 18 samples were analyzed per day. All samples were examined within 20 days. Inoculation carried out one day prior to analysis. Next day the samples were processed like described below and new samples were inoculated. For the purposes of documentation a task plan was made for each day prior to start of main investigations. A protocol was written on the examination day and the results were checked for inhibition / enhancement immediately after running the assay. As an example the plan for day No. 2, the protocol for this day and its results are presented in table 2-6 to 2-8.

TABLE 2-6 ORIGINAL PLAN FOR NUMBERING THE TUBES ON DAY NO. 2

Taskplan for day No. 2		
Cellulose	**Synthetic**	**Synthetic**
Escherichia coli	*Escherichia coli*	*E.coli*
0.5 ml, 200 EU	0.5 ml, 200 EU	0.5 ml, 200 EU
Tween		
Shaking	**sonication**	**shaking**
5 vials with filter		
1vial without filter		
6 vials	6 vials	6 vials
probe No. 1 to 6	probe No. 7 to 12	probe No. 13 to 18
PPC 1 to 6	PPC 7 to 12	PPC 13 to 18

TABLE 2-7 ORIGINAL PROTOCOL FOR DAY NO. 2

Kit-No.: 7L 2670	**Protocol**	**Date: 1/7/98**
H_2O-Lot-No.: 22 7 09 B	**Maininvestigation**	**File: 8107Ro**
	2. Day	

No.	Dilution	Procedure	Others
Std		**Standardcurve** with 0.005% **Tween** 20	
P1-5		P1-5: each ½ **Cellulose fiber filter** with 0.5 ml aqueous ***Escherichia coli*** solution. (approx. 400 EU/ml) inoculated, desiccated overnight under "laminar flow", in 100 ml (0.005% **Tween** 20), **shaking** (1h, 200 rpm), approx. 3 ml filled in tubes, measured in duplicate.	PPC1 to 5: Spike with **Tween,** 5 sec. shaking before adding LAL
P6		Same as above but **without** filter	PPC6: Spike with **Tween,** 5 sec. shaking before adding LAL
P7-11		P7-11: each ½ **Synthetic fiber filter** with 0.5 ml aqueous ***Escherichia coli*** solution. (approx. 400 EU/ml) inoculated, desiccated over night under "laminar flow", in 100 ml (0.005% **Tween** 20), **sonication** (45'), approx. 3 ml filled in tubes, measured in duplicate.	PPC7 to 11: Spike with **Tween,** 5 sec. shaking before adding LAL
P12		Same as above but **without** filter	PPC12: Spike with **Tween,** 5 sec. shaking before adding LAL
P13-17		P13-17: Each ½ **Synthetic fiber filter** with 0.5 ml aqueous ***Escherichia coli*** solution (approx. 400 EU/ml) inoculated, desiccated over night under "laminar flow", in 100 ml (0.005% **Tween** 20), **shaking** (1h, 200 rpm), approx. 3 ml filled in tubes, measured in duplicate.	PPC13 to 17: Spike with **Tween,** 5 sec. shaking before adding LAL
P18		Same as above but **without** filter	PPC18: Spike with **Tween,** 5 sec. shaking before adding LAL

TABLE 2-8 RESULTS OF DAY NO. 2

Standard Curve made with Tween							
Procedure	Measuring		Spiking				
	No.	Value P	No.	Value PPC	PPC-P=	Deviat.	reliable?
CF / E.coli / Tw / S	P1	0.008 EU/ml	PPC1	3.761 EU/ml	3.75 EU/ml	-17%	OK
CF / E.coli / Tw / S	P2	2.309 EU/ml	PPC2	6.084 EU/ml	3.78 EU/ml	-16%	OK
CF / E.coli / Tw / S	P3	0.157 EU/ml	PPC3	3.553 EU/ml	3.40 EU/ml	-25%	OK
CF / E.coli / Tw / S	P4	0.072 EU/ml	PPC4	3.267 EU/ml	3.20 EU/ml	-29%	OK
CF / E.coli / Tw / S	P5	0.086 EU/ml	PPC5	3.359 EU/ml	3.27 EU/ml	-27%	OK
E.coli / Tw / S	P6	0.013 EU/ml	PPC6	3.761 EU/ml	3.75 EU/ml	-17%	OK
SF / E.coli / Tw / So	P7	0.075 EU/ml	PPC7	3.359 EU/ml	3.28 EU/ml	-27%	OK
SF / E.coli / Tw / So	P8	0.043 EU/ml	PPC8	3.178 EU/ml	3.14 EU/ml	-30%	OK
SF / E.coli / Tw / So	P9	0.212 EU/ml	PPC9	4.613 EU/ml	4.40 EU/ml	-2%	OK
SF / E.coli / Tw / So	P10	0.127 EU/ml	PPC10	3.870 EU/ml	3.74 EU/ml	-17%	OK
SF / E.coli / Tw / So	P11	0.056 EU/ml	PPC11	3.454 EU/ml	3.40 EU/ml	-24%	OK
E.coli / Tw / So	P12	0.120 EU/ml	PPC12	3.008 EU/ml	2.89 EU/ml	-36%	OK
SF / E.coli / Tw / S	P13	0.101 EU/ml	PPC13	3.359 EU/ml	3.26 EU/ml	-28%	OK
SF / E.coli / Tw / S	P14	0.054 EU/ml	PPC14	3.359 EU/ml	3.31 EU/ml	-27%	OK
SF / E.coli / Tw / S	P15	0.057 EU/ml	PPC15	2.928 EU/ml	2.87 EU/ml	-36%	OK
SF / E.coli / Tw / S	P16	0.069 EU/ml	PPC16	1.416 EU/ml	1.35 EU/ml	-70%	NO
SF / E.coli / Tw / S	P17	0.164 EU/ml	PPC17	1.484 EU/ml	1.32 EU/ml	-71%	NO
E.coli / Tw / S	P18	0.067 EU/ml	PPC18	3.359 EU/ml	3.29 EU/ml	-27%	OK

F = Synthetic fiber
CF = Cellulose fiber
Tw = Tween 20
S = shaking
So = sonication
P = probe
PPC = positive product control
Deviat. = deviation of spike

All preinvestigations were performed by one person. In the main investigations I have done the organization, inoculation, extraction and analysis every day. This study was performed as a single blind study. A technical assistant did the assay work. The technician did not know which kind of sample she was placing on the microplate.

2.1.5.2 QUALITY CONTROL

All filters were stamped like described in section 2.1.2 on one single day. Out of this big lot 10 pieces of every material arbitrarily selected to determine the basic contamination (see 3.2.2). The rest was used for the main investigations.

The standard curve for standard endotoxin was obtained by linear regression. The coefficient of variation had to be greater than or equal to 0.98 [71]. The slope had to be between -1 and -0.1[71].
When the observed endotoxin activity of a sample with acceptable spike recovery was less than 0.005 EU/ml its value was set as zero. If it was not possible to obtain the smallest standard (0.005 EU/ml) for all measurements which are less than 0.05 EU/ml and reliable were getting the worth of < 0.05 EU/ml.

The microplate reader MRX passed all calibration tests. There was no evidence of any malfunction.

The timetable was set as described above (2.1.5.1) prior to start the investigation. Before starting the criteria for repeating a part of the test were established.

All spikes were measured in duplicate and the difference between duplicates was acceptable if less than 10% of the mean. Duplicate measurement for samples was allowed to deviate more than 10% of the mean because its activities of endotoxin were often small (< 0,1 EU/ml). Even higher percentage deviation of low activity resulted in small total differences. Preinvestigations showed that it is not possible to attain a smaller variance when investigating filter materials (see discussion).

Data were used to compute best method if at least 4 of 5 spike recoveries were in the acceptable range. Thus, one spike out of five probes with the same filter material, extracted in the same manner may exceed ± 50% spike recovery. In this case the failed replicate was deleted and the other four were used to determine the "best method".
If two or more spikes were not reliable, all five samples were diluted 1:10 in the solution media and were assayed again. Only the diluted samples were used to determine the "best method".

2.1.6 DEPYROGENIZATION

To render glassware and all non-disposable items endotoxin free dry heat depyrogenization was used. All non-disposable materials that came in contact with solution or filters were treated in order to make it free of endotoxin. All material was placed in an oven for at least 4 hours at 180°C [85-90].

2.1.7 OTHER NEEDED MATERIALS

Materials needed are listed below.

2.1.7.1 DISPOSABLE MATERIALS

Disposable materials used are described in Table 2-9.

TABLE 2-9 DISPOSABLE MATERIALS

Object	Company
Pipette tips Biopur™ with an endotoxin activity of < 0,002 EE/ml.	Co. Eppendorf
Repetitive pipette tips Biopur™ with an endotoxin activity of < 0,002 EE/ml.	Co. Eppendorf
Microplates Microtest III™ with an endotoxin activity of < 0.5 EU/ml per product	Co. Becton Dickinson Labware

2.1.7.2 NON-DISPOSABLE MATERIALS

Non-disposable materials used are described in Table 2-10.

TABLE 2-10 NON-DISPOSABLE MATERIALS

Object	Company	Depyroge-nization
Repetitive pipette Multipette™ plus	Co. Eppendorf	3
Different pipettes (100µl and 1000µl)	Co. Eppendorf	3
Vortex-Mixer with 2,500 rpm	Co. Janke + Kunkel	3
Some pair of tweezers out of steel		1
Some scissors out of steel		1
Reagent vials with Catalogue No. N207	Co. BioWhittaker	1
Glass tubes for centrifugation with a metal top. Made out of Duran™ (borosilicate glass).	Co. Schott, Germany	1
Measuring cylinder 100 ml Made out of Duran™ (borosilicate glass).	Co. Schott, Germany	1
Incubating rack Single Micro-Hywel™.	Co. Chromogenix	3
A punch type GP 2	Co. Imetex, Netherlands	2
Dishwasher	Co. Miele	3
Oven	Co. Heraeus	3
A microplate reader MRX	Co. Dynatech Laboratories	3
Test Sieve with mesh width = 63 micron	Co. Retsch	3

1) Dry heat for 4 hours at 180°C.

2) Wiped with 60% alcohol (2-Propanol) and heated with a gas flame

3) Not in direct contact with samples

A punch machine type GP 2 (Co. IMETEX Venlo, Netherlands) (high x width x depth = 1m x 1m x 0.4m) with a self-made stamp was used to produce round filter pieces. Punched segments had a diameter of 64 mm. Because of its big size it was not heated in the oven.

A dishwasher was used for cleaning all non-disposable material. After cleaning all equipment was washed twice with distilled water because some influence of the cleaning tensids to the Limulus Amebocyte Lysate assay were observed. Then the clean objects were placed in the oven.

A microplate reader MRX of Co. Dynatech Laboratories. It detected spectrophotometrically the optical density at wave length of 405 nm and a constant temperature of 37°C. Measurements were taken every 10 seconds for a period of 100 minutes.

A Computer, IBM compatible with a DOS operating system, Windows 3.11 and the software Revelation™ Version G 3.04 of Co. Dynex Technologies.

2.1.8 STATISTICAL ANALYSIS

Analyses are divided into a descriptive- and a inferential part. This was made because of the difficulties in interpreting the results. Statistical analyses were performed with "Generalized Linear Models" (GLM) of the SPSS-Software Version 8.0.

2.1.8.1 POSITIVE TEST TUBES

The activity of endotoxin used for contaminating the filter pieces had to be determined. As described in section 2.1.4 it was determined a positive test tube (PTT) for every set of 5 samples. This PTT was set as 100% recovery rate for these five filters and it was named "True amount" (TA 3).
Two other TAs were feasible. First, mean or median of all 3 PTT's made per extraction manner (TA 1) and second, the corresponding PTT which has been done for every 5 filter samples and if there was a second PTT done exactly the same time in the same manner then the mean out of these two (TA 2). Coefficients of correlation for these data were calculated. For results see section 4.6.1.

2.1.8.2 VALUES USED FOR STATISTICAL ANALYSIS

Table 2-11 shows the formulas used for calculating the variance, difference and mean square fractional difference (MSFD). These items were used to describe the statistics of the main investigation see section 3.1.2.

TABLE 2-11 VALUES USED IN TABLE 3-2 TILL TABLE 3-24

Used in Tables	Description	Formulation
Standard deviation (SD)	SD of the fractional differences (%)	$\left(\sqrt{\frac{1}{n}\sum_{i=1}^{n}\left(\left(\frac{1}{m}\sum_{m}^{1}\frac{MV_i - TA_i}{TA_i}\right) - \frac{MV_i - TA_i}{TA_i}\right)^2}\right)\cdot 100$
Difference	Mean of the fractional differences (%)	$\left(\frac{1}{n}\sum_{i=1}^{n}\frac{MV_i - TA_i}{TA_i}\right)\cdot 100$
MSFD	Mean square fractional differences (%)	$\left(\sqrt{\frac{1}{n}\sum_{i=1}^{n}\left(\frac{MV_i - TA_i}{TA_i}\right)^2}\right)\cdot 100$

MV_i = measured value of the i-th sample
TA_i = true amount of the i-th sample
m = number of measurements under same conditions
n = number of means of "m"

SD shows variability of results.
Difference takes into account the different activities of contamination. Positive and negative values can erase each other.
MSFD takes into account the reliability (variance), validity (difference from TA) and the different activities of contamination.

2.2 PRELIMINARY INVESTIGATIONS

This section describes the investigations done to prepare the main investigations. For some preliminary investigations the quantitative chromogenic endpoint tests of Co. "BioWhittaker" and "Endosafe" Charleston, SC, USA were used.

2.2.1 INVESTIGATIONS DONE ON STANDARD CURVES

Dissolved LPS in a) water, b) 0.005% Tween 20 and c) TAP. Then each of the three LPS solutions was diluted to produce a standard curve in the respective diluent (2.1.1.2.1). All standard dilutions of a), b) and c) were transferred to the microplate. Except the highest standard b) and c) dilutions were placed to an extra set of wells which already had been loaded with the spike volume (10 µl of the LPS stock solution dissolved in water (5 EU/10µl)).

Post incubation the plate was measured by the reader with a) as the standard curve and b) and c) as samples with their spikes. Then the plate was recalculated twice for b) and c) as the standard curves, respectively. For results see 3.2.5.

2.2.2 IMPROVEMENT OF SPIKING

When placing a 10µl spike into 100µl sample the distribution of the spike is unpredictable. The spike has a very high activity of 50 EU/ml in a small volume. The sample has in most cases a low activity of endotoxin and a 10 fold larger volume. Placed into the sample the spike will not distribute immediately, the LPS tends to build micelles [84, 91-93]. In the worst case the endotoxin will form big micelle cylinders and will not be detected and therefore underestimated by the LAL test.

To investigate an improvement of spike recovery if the spiked sample is shaken prior to analysis with the reader two activities of LPS (5 EU/ml and 0.5 EU/ml) were produced. Spikes with 5 EU (10 µl of the 50 EU/ml stock solution) were placed into 16 wells of the microplate. 100 µl of 5 EU/ml and 0.5 EU/ml activity were added to 4 wells, respectively. The plate was shaken vigorously in a special mode for 5 seconds by the reader device. Then the missing 4 samples of either activity were added and all samples were incubated for 10 minutes. For Results see 3.2.4.

2.2.3 TWEEN 20 CONCENTRATION YIELDING HIGHEST LAL ACTIVITY

Tween 20 does interfere the Limulus Amebocyte Lysate test and it was aim for investigating a concentration to minimize this effect. LPS (see 2.1.1.1.2) and *Escherichia coli* (see 2.1.3.2.3) endotoxin were diluted in five different concentrations (0.07% – 0.0007%) of Tween 20. With each Tween-concentration four activities (0.15 EU/ml – 1.20 EU/ml) of LPS and one (0.65 EU/ml) of *Escherichia coli* were produced. Unlike main investigations the standard curve was made with water. For results see Table 3-42. A second set of samples was made the same way with six (0.05% – 0.0005%) Tween 20 concentrations and two LPS (2.1.3.2.1) activities (0.41 EU/ml and 0.61 EU/ml). Results are shown in Table 3-43.

2.2.4 DEPYROGENIZATION

To render glassware and all non-disposable items endotoxin free dry heat depyrogenization was used. The destruction of endotoxin by γ-radiation was shown.

2.2.4.1 DRY HEAT

All non-disposable materials that came in contact with solution or filters were treated in order to make it free of endotoxin. All material was placed in an oven for at least 4 hours at 180°C [85-90]. The oven was tested with Endotoxin Challenge Vials™ (ECV) (Cat. No. N187 of Co. BioWhittaker, Walkersville, MD; lot number: 6L 4720) for its validity. The vials containing 500 ng of *Escherichia coli* 055:B5 endotoxin with an estimated activity of more than 1000 EU/ml. For depyrogenation process to be valid it is recommended by the United States Pharmacopeia (USP) that the endotoxin content of a challenge vial must be reduced at least 1,000 fold (> 3 log cycle reduction).

2.2.4.2 STERILIZATION WITH γ-RADIATION

Six ECV (Cat. No. N187 of Co. BioWhittaker, Walkersville, MD; lot number: 6L 4720) (2.2.4.1) were γ-radiated with 60Co in a dose of 25 KGray (2.2.4.4). Three ECV were stored at 4°C as reference (see results).

2.2.4.3 DEPYROGENIZATION WITH DRY HEAT

In order to render the filters free of endotoxin depyrogenization with dry heat was carried out. In the preinvestigations punched filter pieces out of cellulose fiber, glass fiber and synthetic fiber as used in the main investigations were studied. The filters were placed in an oven for at least 4 hours at 180°C. The material structure was inspected with an electron microscope.

2.2.4.4 DEPYROGENIZATION WITH γ-RADIATION

In preinvestigations the depyrogenizing effect of γ-radiation with 25 KGray form a 60 Cobalt device was tested. The radiation was carried out by the Hahn-Meitner-Institute in Berlin. Five punched filter pieces each out of cellulose fiber, glass fiber and synthetic fiber were bisected. The synthetic fiber filters were made of a different and thinner material as the ones used in the main investigations. Both corresponding parts were contaminated with the same activity of endotoxin (1,000 EU of LPS). Each part was heat-sealed in a sterilization package and signed for identification (matching parts). One half was sent to the Hahn-Meitner-Institut in Berlin. The corresponding halves were stored in a refrigerator at 4°C. All samples were analyzed with the KLARE method and the KQCL-Test of BioWhittaker with the Lot No: 7L 3690. The samples were extracted in 50 ml of TAP buffer and were shaken for 1 hour at 200 rpm.