CLB VIRUS SAFETY SERVICES

Final Report

FR3202

Process validation

"Lobator sd-1"

for inactivation of

CPV

Applicant:	Telos Germany, co ltd. medical equipment Unter den Linden 26 6303 HUNGEN - OBBORNHOFEN GERMANY

Test facility:	Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Department Clinical Viro-Immunology Plesmanlaan 125 1066 CX Amsterdam The Netherlands

Code CLB:	*V*-21
Code applicant:	Lob-CPV
Date initiation study:	April 2, 1996
Date completion study:	April 24, 1996
Report date:	May 8, 1996

Assay : *Process Validation Viruses* ("Lobator sd-1")
Code : *V*-21
Code applicant : Lob/CPV

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RESPONSIBILITIES

Design, validation and performance of the disinfection procedures and femoral head maesurement and temperature measurement were the responsibility of the applicant. CLB Virus Safety Services was responsible for virus preparation, virus titrations and biosafety aspects during performance of the study.

The project was carried out in a BSL3 (Biosafety on Level 3, CDC-NIH) classified laboratory at the Department of Clinical Viro-Immunology (Prof. F. Miedema, Head of the Department) at the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service under direction of H. Schuitemaker, Ph.D (Project Manager of CLB Virus Safety Services).

Virus preparations and virus titrations were carried out at the laboratory of Clinical Viro-Immunology of the CLB by L. Berger, M.G. Holthuis, A.M. van der Hulst and E.A. Poelstra under direction of F.G. Terpstra (Manager Operations).

The study was subject to auditing by M.H. Post (Quality Assurance Manager of CLB Virus Safety Services).

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SUMMARY

The thermal disinfection process of Lobator sd-1 was tested for the ability to inactivate canine parvovirus (CPV).

The effectiveness of this disinfection process was calculated by comparison of the amount of virus (cell-free + cell-associated) before treatment and the recovery of virus in the output sample and was expressed as the reduction factor.

At the end of the thermal disinfection process of Lobator sd-1 (92 minutes) no infectious virus was detectable.

The reduction factor (log_{10}) for the disinfection process was:

CPV: $> 5.87^{*} \pm 0.29$

* Since the amount of inoculated virus was $10^{6.38}$ $TCID_{50}$ Units and the detection limit was $< 10^{0.51}$ $TCID_{50}$ Units, the reduction factor was > 5.87.

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1 *INTRODUCTION*

Transplantation of allogenic bone is a well accepted procedure all over the world. Allogenic transplants may be associated with the transmission of viruses, e.g. human immunodeficiency virus, hepatitis B and C virus, and parvovirus B19. The validation of procedures for viral inactivation would be an essential part in establishing the safety of bone transplants in lieu of serological screening tests. When efficient inactivation of relevant or model viruses by the procedure is demonstrated, this procedure is thought to be effective in inactivating any adventitious virus.

The objective of this study was to measure the efficacy of inactivation of canine parvovirus (CPV) by applicant's thermal disinfection process of "Lobator sd-1". CPV was used because it is a well accepted model virus for human parvovirus B19. Furthermore, since resistance to physicochemical treatment of CPV is classified as very high[1,2], it may be indicative for the capabilities of the thermal disinfection process. Therefore CPV could serve as a general model for viruses with a comparable or lower resistance to inactivation like hepatitis B virus (medium resistance).
The protocol included a kinetic study of the inactivation of CPV by the process.

The protocol of this study conforms to the requirements described in "Validation of virus removal and inactivation procedures" from the Commission of the European Communities (III/8115/89-EN, Final 1991) and the German Federal requirements "Requirements of validation studies for demonstrating the virus safety of medicinal products derived from human blood plasma" (Bundesanzeiger Nr. 84, Mai 1994).

[1]Note for guidance on virus validation studies: the design, contribution and interpretation of studies validating the inactivation and removal of viruses.
CPMP Biotechnology Working Party (CPMP/BWP/268/95, Final).
[2]Blood Safety & Screening, October 23-25, 1995, Washington
Practical Considerations in the Performance and Validation of Viral Clearance for Human Blood and Plasma Products, J.A. Boose

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2 *MATERIALS*

2.1 Test samples

The materials (see appendix 1) used in the disinfection process were supplied by the applicant. The samples were delivered on the day of the experiment.

2.2 Reagents and equipment

Reagent and equipment supplied by the applicant and used in the disinfection process are listed in appendix 2 and 3.

2.3 Model viruses

Canine parvovirus (CPV), strain 780916, a non-enveloped DNA virus (size: 20 nm).

The virus is kept as validated stock at CLB.

2.4 Cell lines

Canine fibroma-derived (A72) cells are used for the preparation of the CPV stock and CPV infected cells, and for the titration assays.

The cell line is kept as validated cell bank at CLB.

2.5 Culture Media

A72 cells were grown in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% foetal calf serum (FCS), penicillin (100 IU/ml) and streptomycin (100 μg/ml).

3 *METHODS*

All reagents and media used in the experiments were sterile. All equipment which was brought into direct contact with the materials had been sterilized.

3.1 Preparation of cell-free CPV

Cell-free CPV was prepared in A72 cells. Uninfected cells were mixed with CPV (MOI=0.01) in culture medium and this mixture was transferred to a tissue culture flask. Cells were allowed to adsorb for 1 hour at 37°C and subsequently culture medium was added. Inoculated A72 cells were cultured for 4 days at 37°C. At day 4 after infection the virus was harvested by one cycle of freeze/thawing. The suspension was transferred into 50 ml tubes and centrifuged for 15 min at 1000xg in order to remove cell debris. Then the supernatant containing the virus was harvested, frozen and stored at -70°C.

3.2 Preparation of cell-associated CPV

At day 0 uninfected cells were cultured in a tissue culture flask at 37°C. At day 1, the monolayer of cells was inoculated with CPV at high multiplicity of infection. At day 2 the infected A72 cells were treated with trypsin/EDTA, collected and pelleted by centrifugation for 10 min at 350xg. The supernatant was removed and the cell pellet was resuspended in a small volume of culture medium. Finally the concentration of resuspended cells was determined.

3.3 Preparation and measurement of femoral heads

Femoral heads were prepared by the applicant; holes were drilled into the spongiosa of the femoral heads to allow insertion of a PCR tube, so that the PCR tube could be located in the center of the femoral head. Each femoral head was measured in two planes after all cartilage was removed. The first measurement (M1) in the coronal plane was taken from the most proximal point of the femoral head to the distal end of the femoral neck (transverse cut across the neck during removal of the femoral head from the patient). The second measurement (M2) in the transverse plane was taken across the diameter of the femoral head at its thickest part. This measurement was taken in several axes in the transverse plane and the minimum value was used.

3.4 Temperature measurement in the femoral heads

The temperature at the core of each femoral head was recorded during processing with the Lobator sd-1 for each experiment. A mobile programmable PC-supported measurement system with 4 channels, battery supported 32 kB memory and a V24/RS232 connector for on line data retrieval with a PC was used. Four Ni-Cr-Ni insulated thermal electrodes with a diameter of 0.5 mm and a length of 100 mm and a precision of 0.1°C were used as temperature sensors. The results of the temperature measurents were recorded on line on a PC with the support of a special IBM compatible software program. The results were stored as graphs and tables.
Prior to use, each thermal electrode was calibrated in ice (0°C) and at room temperature (± 22°C). One electrode was used per experiment. The electrode was inserted into the center of the femoral head along side the PCR tube. Then the hole was sealed with a plastic lid and waterproof two component silicone glue so that no Ringer's lactate solution could leak through the hole into the bone.

3.5 Spiking of the femoral heads and performance of the inactivation process

Prior to use in the experiment, the frozen femoral heads were thawed and adjusted to room temperature in a water bath and kept at this temperature for 2 hours. A mixture of cell-free CPV (batch 4) and cell-associated CPV (final concentration 10^6 cells/ml) in culture medium containing approximately 50% serum was prepared. A volume of 2.4 ml of this mixture was divided in six PCR tubes. The maximum volume of virus-containing mixture that could be used in the study was limited by the size of the PCR tubes. In turn the maximum size of the PCR tubes used was limited by a concern to avoid removing an excessive amount of cancellous bone from the femoral heads. One sample of the mixture was titrated immediately and was labelled Lob-CPV. Another sample was kept at room temperature for 92 minutes

and was used as a bench control (label: Lob-BC-CPV-92). Five PCR tubes and 5 thermal electrodes were placed in the holes of five different femoral heads and thereafter parafilm was used to seal and cover the lids of the PCR tubes. Onto the PCR tubes a silicon glue was polymerized to prevent any access of liquid. Then the femoral heads were deposited into a polypropylene container filled with 250 ml of sterile Ringer's lactate. The heating program of the Lobator sd-1 was started with continuous stirring of the Ringer's lactate. After 30, 45, 62, 75 and 92 minutes (complete process cycle) a femoral head was instantly taken out of the Ringer's lactate. The PCR tube was withdrawn from the femoral head and the contents of the PCR tube were titrated within 2.2 minutes after removal from the Ringer's lactate. For this purpose the 0.4 ml sample from the PCR tube was diluted in 24 ml of culture medium. From this mixture of 24.4 ml the dilutions as mentioned in Table 1 were made.

The samples were labelled:

Lob-CPV (virus/cell-mixture)
Lob 1-CPV-30*
Lob 2-CPV-45*
Lob 3-CPV-62*
Lob 4-CPV-75*
Lob 5-CPV-92*
Lob-BC-CPV-92 (bench control)

* These samples were also tested in bulk cultures.

3.6 Control samples

For the calculation of the reduction factors, the viral titre as determined in the mixture of cell-free CPV and cell-associated CPV (Lob-CPV) was used.

3.7 $TCID_{50}$ titration assays

To measure the CPV $TCID_{50}$ values of the collected samples, the CPV-susceptible cell line A72 was used. A volume of 100 μl of an A72 cell suspension (0.01 x 10^6 cells/ml culture medium) was added to each well of a 96-wells flatbottom microtitre culture plate. After incubation in a humidified incubator with 5% CO_2 at 37°C for one day, the cells were adhered to the bottom of the wells. From the output samples a twelve-step threefold serial dilution in culture medium was made. From each dilution step of the series, 8-plo 50 μl volumes were added to the wells with the adherent cells. The microtiter culture plates were incubated for 7 days and the cultures were examined microscopically for the emergence of CPE.

$TCID_{50}$ was defined as the reciprocal dilution that was able to infect 50% of the inoculated cultures under the conditions described above.

3.8 Testing for virus presence in a bulk system

Simultaneously and under the same conditions as the titration assay, larger volumes of the output samples were tested in 175 cm^2 tissue culture flasks in a volume of 180 ml, for the

presence of virus which might result in a lower detection limit. When no residual virus was found in the output samples when tested in the titration assay, the results of these culture flasks were used for the calculation of the viral titre and reduction factor.

4 *CALCULATION OF RESULTS*

The viral titre ($TCID_{50}$/ml) was calculated according to the formula:

$M = X_k + \frac{1}{2}d - (d \times \text{Sum } p_i) + v$
where: M = log of the viral titre
X_k = log of the highest dilution that established infection in all replicates
d = log of the dilution factor
p_i = ratio of positive replicates/number of replicates, starting from the highest dilution that established infection in all replicates
v = log of the testvolume/well or flask (ml)

In case the lowest dilution did not establish infection in all replicates, the assumption was made that the dilution that theoretically would have come directly before the lowest dilution tested would have established infection in all replicates ($TCID_{50}$/ml $\leq$ calculated value).

In case the highest dilution still showed infection in one or more replicates, the assumption was made that the dilution that theoretically would have come directly after the highest dilution tested would have shown no infection ($TCID_{50}$/ml $\geq$ calculated value).

The standard error was calculated according to the formula:

$S_m = \pm \sqrt{[d^2 \times \text{Sum } \{p_i(1-p_i)/(n_i-1)\}]}$
where: S_m = standard error of the viral titre
d = log of the dilution factor
p_i = ratio of positive replicates/number of replicates, starting from the highest dilution that established infection in all replicates
n_i = number of replicates

The 95% confidence limit was calculated according to the formula:

95% CL = $S_m \times 1.96$
where: 95% CL = 95% confidence limit
S_m = standard error of the viral titre

In case of complete inactivation the viral titre (upper 95% confidence limit) was calculated according to the formula:

$TCID_{50}$/ml = log{(-ln 0.05)/total testvolume}

In case complete inactivation was found in the titration assay and incomplete inactivtion was found in the bulk culture, the minimal virus titre was calculated according to the formula:

$TCID_{50}/ml \geq \log\{1/testvolume\}$

The viral reduction factor was calculated according to the formula:

$$10^{rf} = \frac{\text{total infectious particles added}}{\text{total infectious particles recovered}}$$

The 95% confidence limit of the reduction factor was calculated according to the formula:

95% CL of reduction factor $= \sqrt{\{(95\% \text{ CL before treatment})^2 + (95\% \text{ CL after treatment})^2\}}$
where: 95% CL = 95% confidence limit

5 *CRITERIA*

5.1 A culture was considered positive if:

A72 cells, growing in a confluent monolayer, showed CPE resulting in detachment of the cells from the bottom of the culture wells.

5.2 The assay was considered acceptable if:

At least 50% of the cultures of each dilution required for calculation of $TCID_{50}$ values could be interpreted.

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6 *RESULTS*

The number of positive cultures are shown in Table 1. Viral titres, standard errors and 95% confidence limits are shown in Table 2. Reduction factors (rf) and their 95% confidence limits are shown in Table 3.
The results of the temperature measurements are shown in Appendices 4-8. The results of the femoral head measurements and the Lobator sd-1 identification are shown in Appendix 9.
The results of the kinetic study of the inactivation of CPV by the process are shown in Figure 1.

Table 1: Number of positive cultures out of a total of 8 replicates per dilution step*

Lob-CPV												
Dilution 3^{-}	3	4	5	6	7	8	9	10	11	12	13	14
Positive cultures	8	8	8	8	5	3	1	1	0	0	0	0
Lob 1-CPV-30												
Dilution 3^{-}	0	1	2	3	4	5	6	7	8	9	10	11
Positive cultures	8	8	8	8	8	8	8	7	4	2	0	0
Lob 2-CPV-45												
Dilution 3^{-}	0	1	2	3	4	5	6	7	8	9	10	11
Positive cultures	8	8	8	8	8	8	6	4	0	0	0	0
Lob 3-CPV-62												
Dilution 3^{-}	0	1	2	3	4	5	6	7	8	9	10	11
Positive cultures	0	0	0	0	0	0	0	0	0	0	0	0
Lob 4-CPV-75												
Dilution 3^{-}	0	1	2	3	4	5	6	7	8	9	10	11
Positive cultures	0	0	0	0	0	0	0	0	0	0	0	0
Lob 5-CPV-92												
Dilution 3^{-}	0	1	2	3	4	5	6	7	8	9	10	11
Positive cultures	0	0	0	0	0	0	0	0	0	0	0	0
Lob-BC-CPV-92												
Dilution 3^{-}	3	4	5	6	7	8	9	10	11	12	13	14
Positive cultures	8	8	8	8	5	1	0	0	0	0	0	0

* Unless indicated otherwise

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Table 2: Calculated values

Sample	$TCID_{50}$/ml	Standard error	95% Confidence limit
Lob-CPV (virus/cell-mixture)	$10^{6.78}$	0.15	0.29
Lob 1-CPV-30	$10^{6.96}$	0.13	0.26
Lob 2-CPV-45	$10^{6.31}$	0.12	0.23
Lob 3-CPV-62[1]	$<10^{2.66}$		
Lob 3-CPV-62[^]	$<10^{0.91}$		
Lob 4-CPV-75[1]	$<10^{2.66}$		
Lob 4-CPV-75[B]	$<10^{0.90}$		
Lob 5-CPV-92[1]	$<10^{2.66}$		
Lob 5-CPV-92[^]	$<10^{0.91}$		
Lob-BC-CPV-92 (bench control)	$10^{6.55}$	0.11	0.21

☐ = not applicable

[1] Calculation of the $TCID_{50}$/ml: log{(ln 0.05)/(0.4/61)}.
[^] A volume of two times 11.3 ml was tested in a bulk culture and no infectious virus was detectable. Calculation of the $TCID_{50}$/ml: log{(ln 0.05)/(22.6/61)}.
[B] A volume of two times 11.5 ml was tested in a bulk culture and no infectious virus was detectable. Calculation of the $TCID_{50}$/ml: log{(ln 0.05)/(23.0/61)}.

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Table 3: Viral reduction factors

Sample	Reduction of virus						
	Virus recovered $TCID_{50}$ Units/ml	Volume of sample (ml)	Fraction of virus inoculum*	Virus inoculated $TCID_{50}$ Units	Total $TCID_{50}$ Units recovered	Reduction factor	95% Confidence limit
Lob-CPV (virus/cell-mixture)	$10^{6.78}$	0.4			$10^{6.38}$		0.29
Lob 1-CPV-30	$10^{6.96}$	0.4	1.0	$10^{6.38}$	$10^{6.56}$	-0.18	0.39
Lob 2-CPV-45	$10^{6.31}$	0.4	1.0	$10^{6.38}$	$10^{5.91}$	0.47	0.37
Lob 3-CPV-62	$<10^{2.66}$	0.4	1.0	$10^{6.38}$	$<10^{2.26}$	>4.12	0.29
Lob 3-CPV-62^	$<10^{0.91}$	0.4	1.0	$10^{6.38}$	$<10^{0.51}$	>5.87	0.29
Lob 4-CPV-75	$<10^{2.66}$	0.4	1.0	$10^{6.38}$	$<10^{2.26}$	>4.12	0.29
Lob 4-CPV-75[B]	$<10^{0.90}$	0.4	1.0	$10^{6.38}$	$<10^{0.50}$	>5.88	0.29
Lob 5-CPV-92	$<10^{2.66}$	0.4	1.0	$10^{6.38}$	$<10^{2.26}$	>4.12	0.29
Lob 5-CPV-92^	$<10^{0.91}$	0.4	1.0	$10^{6.38}$	$<10^{0.51}$	>5.87	0.29
Lob-BC-CPV-92 (bench control)	$10^{6.55}$	0.4	1.0	$10^{6.38}$	$10^{6.15}$	0.23	0.36

☐ = not applicable

* Ratio of volume of virus inoculum present in sample and volume of virus inoculated in starting material.
^ A volume of two times 11.3 ml was tested in a bulk culture and no infectious virus was detectable.
[B] A volume of two times 11.5 ml was tested in a bulk culture and no infectious virus was detectable.

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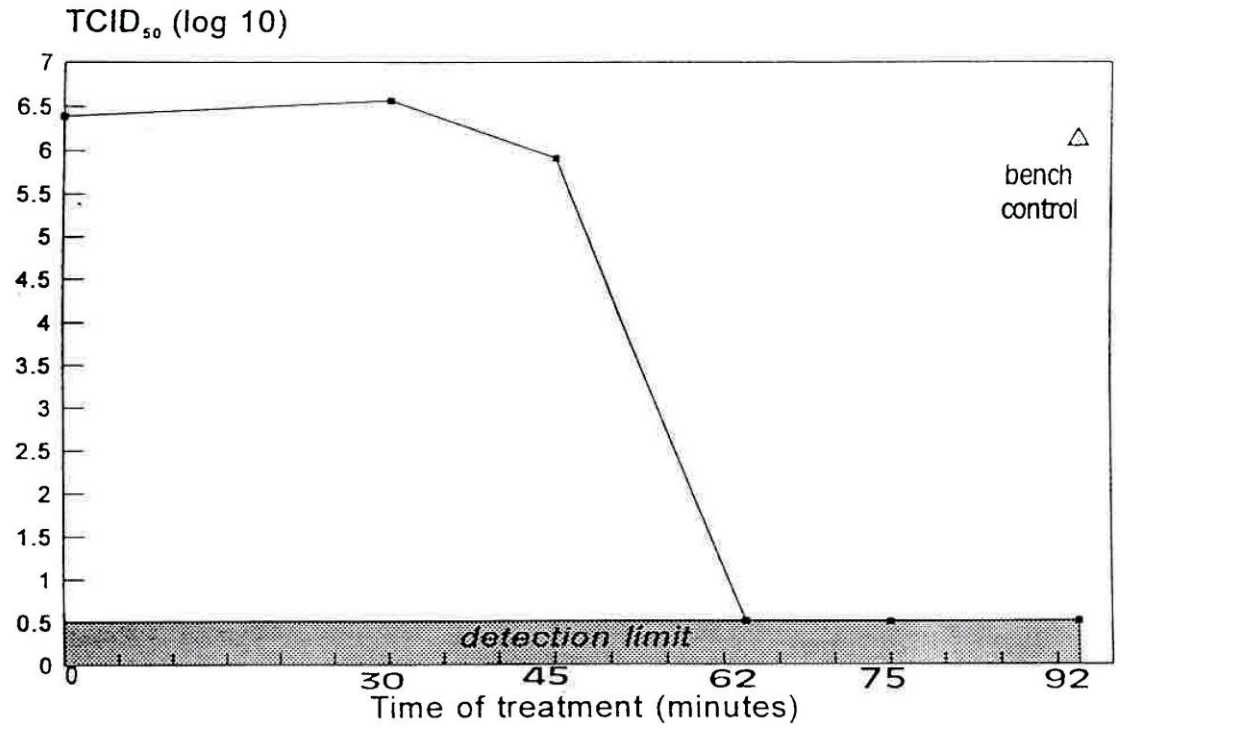
Figure 1: Kinetic study of CPV inactivation



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7 *CONCLUSIONS*

The study was valid; the criteria were met.

At the end of the thermal disinfection process of Lobator sd-1 (92 minutes) no infectious virus was detectable.

The reduction factor (log_{10}) for the disinfection process was:

CPV: $> 5.87^{*} \pm 0.29$

* Since the amount of inoculated virus was $10^{6.38}$ $TCID_{50}$ Units and the detection limit was $< 10^{0.51}$ $TCID_{50}$ Units, the reduction factor was > 5.87.

8 *RECORD MAINTENANCE*

Protocol, modifications, a copy of the reports and all correspondence between CLB Virus Safety Services and the applicant will be maintained within a file in the CLB. These records will be retained for a period of ten years following submission of the final report to the applicant.

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9 *APPROVALS*

11/06/96

F.G. Terpstra
Manager Operations

Date

Schuitemaker

11 06 96

Dr. H. Schuitemaker
Project Manager

Date

10 *QUALITY ASSURANCE STATEMENT*

This final report has been audited by the Quality Assurance Manager of CLB Virus Safety Services and has been found to describe the methods used and to reflect the raw data of the study.

Mupost

110696

M.H. Post
Quality Assurance Manager

Date

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Appendix 1: List of samples obtained from applicant

Starting materials

Sample name	Amount of sample obtained	Sample designation
Femoral heads	5 pieces	Lob-1-5 CPV (V-21B)

Appendix 2: Reagents supplied by applicant

- Ringer's lactate (sterile)

Appendix 3: Equipment supplied by applicant

- Lobators sd-1, serial numbers 1090, 1093 and 1142
- PC with IBM compatible software (Online 2, version 4.32, DEMA Software GmbH)
- PCR tubes with caps (size: 0.45 ml)
- Silicon glue, two component
- Sterile disinfection container, complete
- Temperature sensors, four Ni-Cr-Ni insulated thermal electrodes with a diameter of 0.5 mm and a length of 100 mm (T430, Ahlborn GmbH, Holzkirchen, Germany), precision 0.1°C
- Therm 2281-8, Ahlborn Mess- und Regelungstechnik GmbH, Holzkirchen, Germany with 4 channels, battery supported 32 kB memory and a V24/RS232 connector

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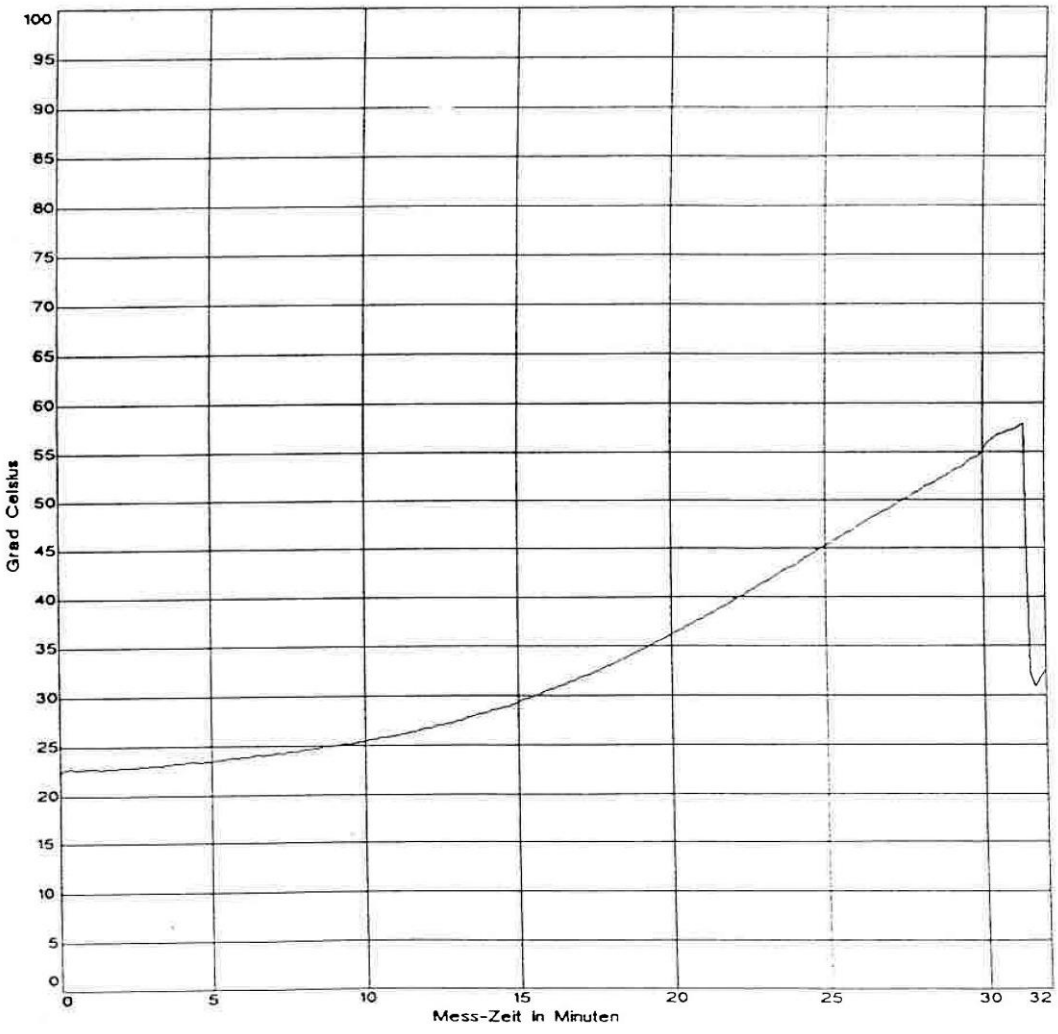
Appendix 4: Temperature measurement of Lob 1-CPV-30



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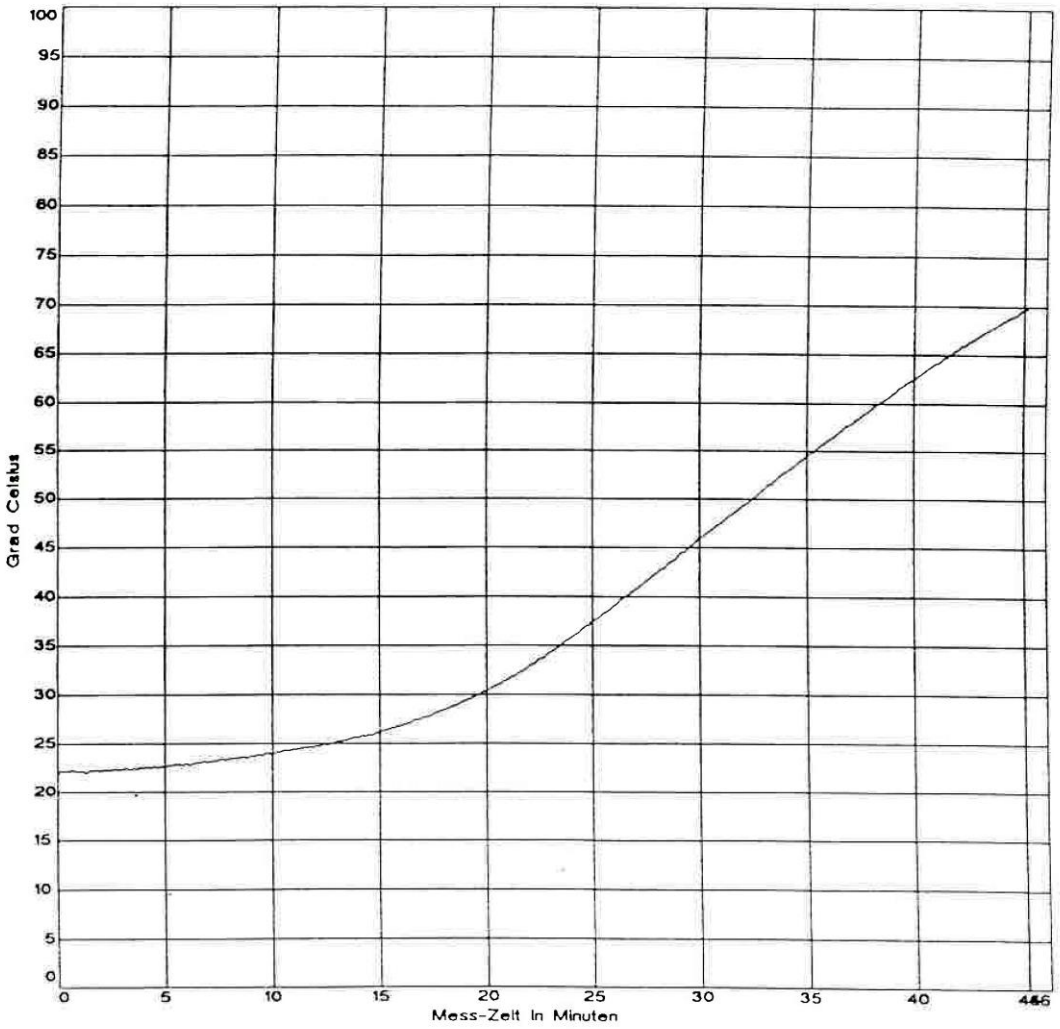
Appendix 5: Temperature measurement of Lob 2-CPV-45



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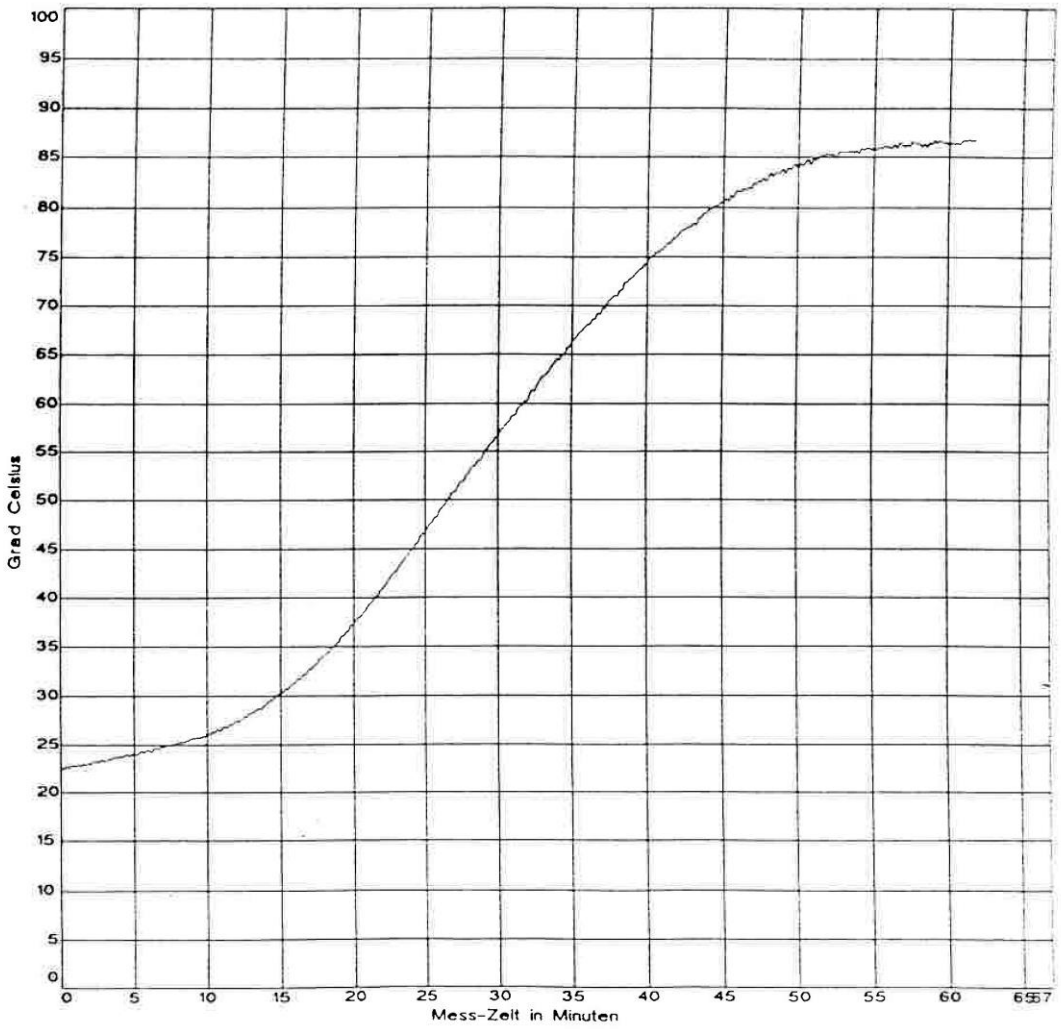
Appendix 6: Temperature measurement of Lob 3-CPV-62



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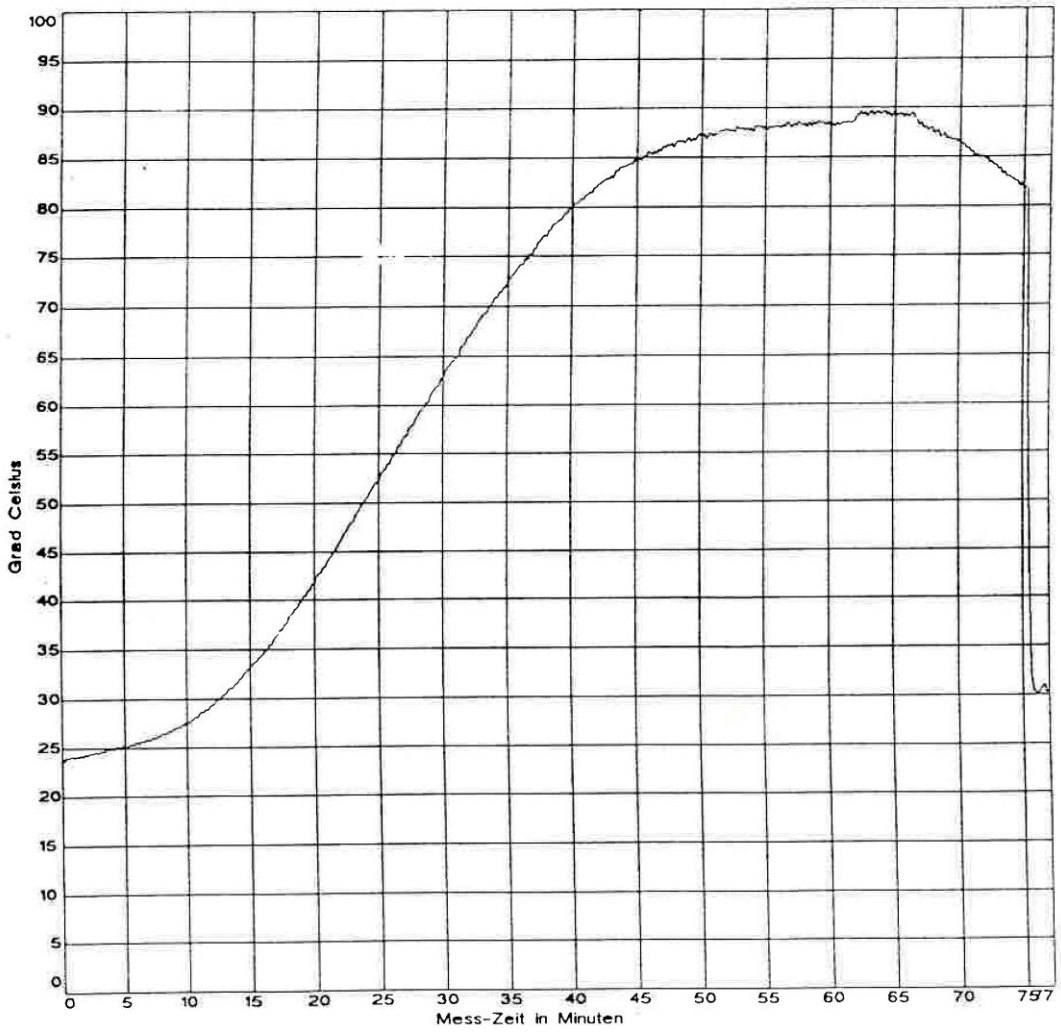
Appendix 7: Temperature measurement of Lob 4-CPV-75



Note: Disturbance at 62 minutes seen on curve is due to the transfer of the disinfection container from the warming unit to the cooling unit

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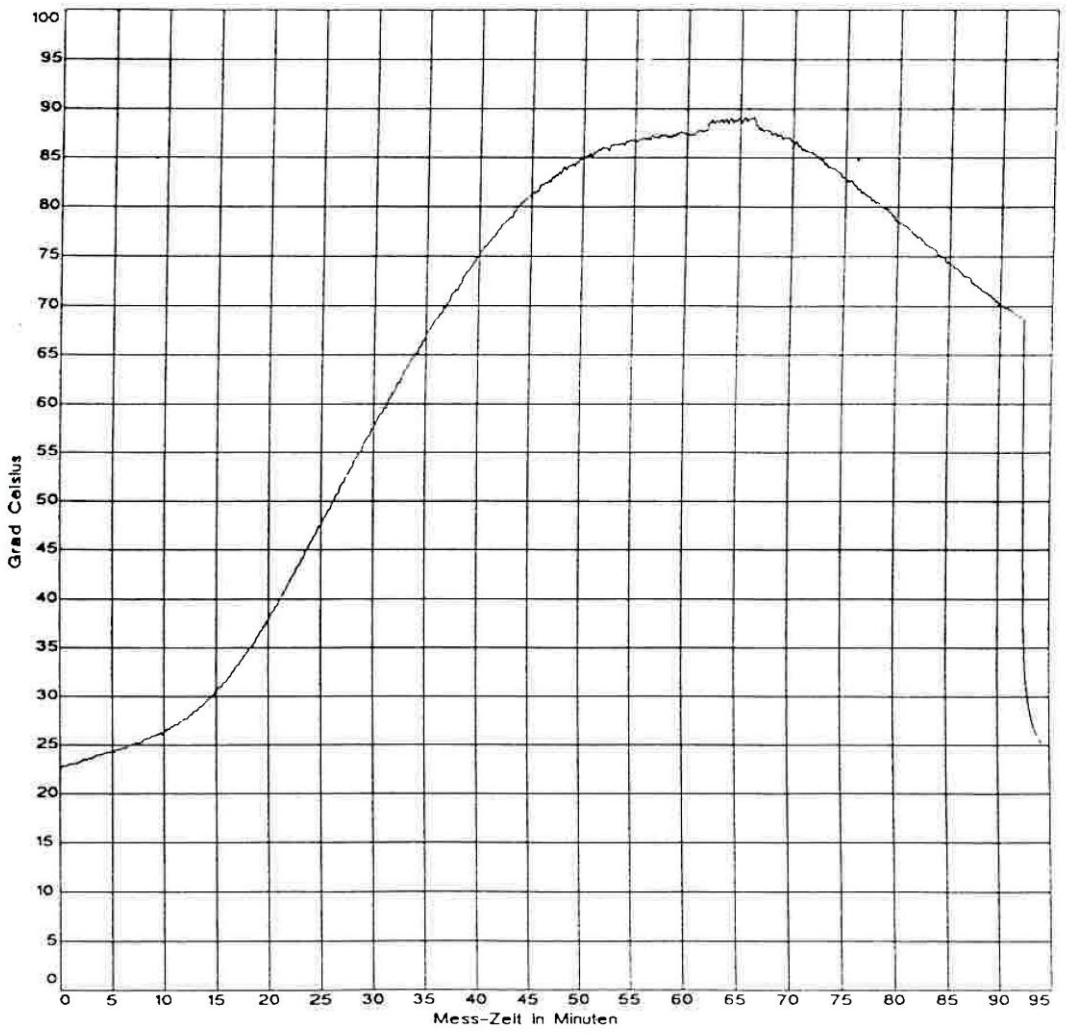
Appendix 8: Temperature measurement of Lob 5-CPV-92



Note: Disturbance at 62 minutes seen on curve is due to the transfer of the disinfection container from the warming unit to the cooling unit

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Appendix 9: Measurement of femoral head

Femoral head measurements and Lobator sd-1 identification

Sample	M1 (mm)	M2 (mm)	Lobator sd-1 (batch number)
Lob 1-CPV-30	48	56	1090
Lob 2-CPV-45	56	55	1093
Lob 3-CPV-62	46	53	1093
Lob 4-CPV-75	50	48	1142
Lob 5-CPV-92	51	47	1090