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Validation of inactivation of bovine viral diarrhoea virus by heat treatment in the manufacturing process of lipoprotein

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Abbreviations

BVDV	Bovine Viral Diarrhoea Virus
MTP	micro titer plate
TCID50	Tissue culture infectious dose 50
SAA	Standardarbeitsanweisung
SOP	Standard Operating Procedure
ATCC	American Type Culture Collection
ECACC	European Collection of Animal Cell Cultures
RNA	Ribonucleic acid
MCB	master cell bank
WCB	working cell bank
CPE	Cytopathic effects
GLP	Good Laboratory Practice

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1 Information on the test

1.1 Title

"Validation of inactivation of Bovine Viral Diarrhoea Virus by Heat Treatment in the manufacturing process of Lipoprotein"

1.2 Purpose of the study

In order to demonstrate the safety of pharmaceutical proteins derived from biological sources it is mandatory for the manufacturer of such products to demonstrate the effective inactivation and/or removal of pathogenic viruses during the manufacturing process. Usually, this is done by the deliberate spiking of a down-scaled version of the manufacturing process with relevant and/or model viruses.
The purpose of this study was to test for the effectiveness of Heat Treatment to inactivate viruses during the manufacturing process of a Lipoprotein. The appropriate model virus for this process is BVDV, an enveloped RNA Flavivirus.

The study was conducted according to "Anforderungen an Validierungsstudien zum Nachweis der Virussicherheit von Arzneimitteln aus menschlichem Blut oder Plasma (Paul Ehrlich Institut and Bundesgesundheitsamt; May 4, 1994)" (1), the "Note for Guidance on Virus Validation Studies (CHMP/BWP/268/95, Final Version 2; February 29, 1996) (2), the "Note for Guidance on Quality of Biotechnological Products: Viral Safety Evaluation of Biotechnology Products derived from Cell Lines of Human or Animal Origin" (CHMP/ICH Q5A/295/95, Final Version, October 1997) (3), and the "Note for Guidance on Plasma Derived Medicinal Products (CHMP/BWP/269/95, Revision 3, January 25, 2001)" (4).

The Sponsor was responsible for all aspects of the scaling-down of the analysed manufacturing process steps

2 Summary

The capacity of heat treatment in the manufacturing process of Lipoprotein to inactivate BVDV was validated. For this purpose, the corresponding process intermediate was spiked with a high-titer preparation of Bovine Viral Diarrhoea Virus (BVDV). The spiked Test Item was subjected to the inactivation procedure. In parallel, the spiked Test Item was kept at room temperature. Samples were withdrawn before, during and at the end of both assays and analysed for the viral titer.
The following table summarizes the log10 reduction factors determined for both assays mentioned above:

Table 1: Summary of the achieved $\log_{10}$ reduction factors

	Heat Treatment (80 °C after 103 min.)	Room temperature after 115 min. [1] / 111 min. [2]	Reduction induced by Heat Treatment only
	A *	B **	C ***
Maximum	4.10	1.47	2.65
Minimum	3.88	1.23	2.63

[1] for run 1

[2] for run 2

* Influence of the spiked Test Item subjected to Heat treatment

** Influence of the spiked Test Item at room temperature

*** Influence of the Heat treatment without any influence of the Test Item (C = A - B)

BVDV was considerably inactivated by the Test Item itself.
The reduction factor determined at the end of the heat treatment is the result of both effects the inactivation by heat and the inactivation by the Test Item itself.

3 Information on the Test Items

For the pre-testing aliquot was defined as Test Item A. The load material for the validation process was defined as Test Item 1.

3.1 Test Item for pretesting

The toxicity and interference assays for Test Item A were performed. The data were defined in study 2/06.166 (performed by NewLab BioQuality AG, Cologne) and were used in this study.

Designation of Test Item A:	Process intermediate before Heat Treatment
Active ingredient:	Lipoprotein
Batch no.:	PT-ABL-0311-LWP
Manufacturer:	Selborne Biological Services Ltd.
Date of manufacturing:	September 2006
Expiry date:	September 2008
Storage condition:	2 - 8 °C
Amount:	1 x 30 mL (was already available at NewLab; retain sample from study 2/06.166)

3.2 Test Items for process validation studies

Designation of Test Item 1:	Process intermediate before Heat Treatment
Active ingredient:	Lipoprotein
Batch no.:	PT-ABL-0311-LWP
Manufacturer:	Selborne Biological Services Ltd.
Date of manufacturing:	September 2006
Expiry date:	September 2008
Storage condition:	2 - 8 °C
Amount:	2x 50 mL (was already available at NewLab; retain sample from study 2/06.166)

Safety measures for laboratory personnel and waste disposal:
In combination with the test systems according to safety instruction as laid down in "Infektionsschutzgesetz", "Tierseuchenerreger-Verordnung", "Biostoffverordnung" and "Gefahrstoffverordnung, § 20" the remaining pretesting samples and Test Items were inactivated and discarded.

4 Test Method

4.1 Principle of the Test Method

It was the purpose of the study to investigate the effectiveness of Heat Treatment to inactivate BVDV during the manufacturing process of Lipoprotein. The Test Item was spiked with the virus at defined titer and then subjected to the virus inactivation procedure. Samples were withdrawn from the spiked Test Item as well as the process samples and monitored for virus by endpoint titration and by bulk analysis (large volume plating). The reduction factors achieved were calculated.

4.2 Justification for the Test System and the process steps to be validated

According to the "Note for Guidance on Virus Validation Studies" (2) the following model viruses were used in this study:
- Bovine Viral Diarrhoea Virus (an enveloped Flavivirus)

Heat treatment has previously been shown to be an effective step in the inactivation of enveloped and non-enveloped viruses.

4.3 Characterisation of the Test System

All viruses are qualified. A master virus stock was prepared from the aliquot provided by the supplier mentioned in the tables. The master virus stock was characterised, e.g. by sequence analysis, growth kinetic, stability and the capability to infect different kind of indicator cells (phenotypic analysis). The master virus stock was used to prepare an intermediate virus stock. All virus working lot's used in the study were prepared from the intermediate virus stock and/or the master virus stock. Documentation according to the GLP requirements ensured the traceability of each working lot to the original virus solution.

The indicator cells were prepared from the cell aliquot provided by the supplier mentioned in the tables. A master cell bank (MCB) and a working cell bank system (WCB) was established. The MCB was well characterised. The absence of mycoplasma was demonstrated by the broth culture assay and the fluorescence assay performed according to the Pharm. Eu. 2002.

The cells and the viruses used in this study are shown in the table below. All data such as the actual passage number of the indicator cells and the specific viral lot no. were documented in the corresponding laboratory documentations.

4.3.1 Viruses and indicator cells

Bovine viral Diarrhoea virus (BVDV)	
Strain	Field isolate of the Robert-Koch-Institute
Origin/Supplier	Robert-Koch-Institute
Characteristics	single stranded RNA virus, enveloped 40-60 nm
Detection/Propagation cells	KL-2, Fetal bovine lung cells
Origin/Supplier	Robert-Koch-Institute
Determination of CPE	after 5-8 days

4.3.2 Cultivation of the Test System

Cultivation of adherent cell lines
Cells were subcultivated once or twice a week. The cells were seeded subconfluently into cell culture flask and incubated at 37.0 °C ± 0.5 °C and 5.0 % ± 0.5 % CO2 in a humidified atmosphere for a cell specific incubation period. After reaching confluence cells were trypsinated, centrifuged, counted and seeded into fresh culture flasks at a defined cell density.

Cells were cultivated according to standard cell culture conditions:

KL-2 cells were kept in DMEM cell culture medium, supplemented with 5 % fetal calf serum, and 50 U/mL penicillin and 50 pg/mL streptomycin.
Preparation of virus stock solutions:
Virus stocks were prepared from infected cell cultures. These cells were frozen and thawed once prior to the virus harvest. Therefore, these viral preparations include cellular components as well as the culture medium which contains 5-10 % (v/v) fetal calf serum. The virus-containing

supernatants was centrifuged at 2000 ± 100 rpm for 10 ± 2 minutes and then filtered using a 0.45 pm filter to remove cell debris. The virus filtrates were stored at -75 to -85 °C in aliquots until use. The titers of the virus stocks were determined in duplicate according to the Spearman-Karber method (6, 7).

5 Determination of the Virus Titer

5.1 Evaluation of virus replication

For the determination of the virus titers of the virus stock, the spiked Test Item, and the process samples serial threefold dilutions were made with cell culture medium. 100 µL aliquots of each dilution were added to 8 wells of a 96 well microtiter plate with adherent cells (in 100 µL cell culture medium per well).

The cells were cultivated in an incubator at 37 °C ± 0.5 °C and 5 % CO2 ± 0.5 % CO_2, in a humidified atmosphere. After an appropriate incubation period cells were inspected microscopically for virus-induced changes in cell morphology (syncytia formation, cytopathic effect). The viral titer that statistically leads to the infection of 50 % of all parallel cultures ($TCID_{50}$) was calculated according to the formula of Spearman and Kaerber (6, 7).

5.2 Calculation and statistical analysis of viral titers

5.2.1 Calculation of the $TCID_{50}$

The virus titer which causes a positive result in 50 % of the infected cultures (TCID50) was calculated according to the method of Spearman and Karber (mode A of calculation):

$$TCID_{50} = 10^{Y_0 - d/2 + d/n \cdot \sum Y_i}$$

Y_0: positive exponent of the highest sample dilution with positive test results in <u>all</u> parallel cultures
d: logarithm of dilution step (3)
n: number of determinations (8)
Y_i: total number of all positive samples starting with Y_0

<u>Calculation of the standard deviation</u>

$$s_e = d\sqrt{\sum \frac{p_i\,(1-p_i)}{(n_i-1)}}$$

s_e: standard deviation
p_i: observed reaction rate
n_i: number of determinations (8)
d: logarithm of dilution step (3)

Calculation of the 95 % confidence limits

$$c_{95\%} = \pm 2\ s_e$$

$c_{95\%}$: 95 % confidence limits
s_e: standard deviation

5.2.2 Probability of detection of virus at low concentrations

For samples where even the highest dose tested in the endpoint titration did not result in the infection of all parallel cultures, the virus titer was calculated as follows:
Assuming the worst case, these samples contain sufficient virus to infect all parallel cultures in that dose from which the first serial dilution for the titration was prepared. In the tables provided in this report, this dose is printed in italic merely to indicate that the sample was not actually tested at this dose (mode B of evaluation).

5.3 Bulk Analysis

The detection limit of a sample analysed for the viral load is defined by the total volume incubated with the indicator cells.
In order to improve the detection limit of a sample, a large volume of one non-toxic and non-interfering concentration of the sample was incubated with the indicator cells (large volume plating). For this, 200 µL of the non-toxic and non-interfering dilution of the sample were added to a defined number of wells containing the indicator cells in 100 µL cell culture medium.
The cells were cultivated in an incubator at 37 °C ± 0.5 °C and 5 % CO_2 ± 0.5 % CO_2 in a humidified atmosphere. After an appropriate incubation period cells were inspected microscopically for virus-induced changes in cell morphology (syncytia formation, cytopathic effect).

5.3.1 Calculation of Bulk analysis

The virus titer for samples where the $TCID_{50}$/mL is zero can be determined by using the Poisson distribution at the 95 % upper confidence limits (mode C of calculation).

$$p = e^{-cv}$$

$$\Rightarrow \quad c = \ln p / -v$$

p: 0.05
c: concentration of infectious particles per mL
v: tested sample volume

5.3.2 Probability of detection of virus at low concentrations in the bulk analysis

The viral titer in samples where the bulk analysis resulted in few single positive wells (< 15 % of all wells) were calculated according to the following formula (mode D of calculation):

$$\text{Titer/mL} = D * (-\ln (W-P) / W) / V$$

D	pre-dilution factor of the sample	P number of virus-positive wells
W	number of all wells tested	V volume per well (0.2 mL)

The virus titer in samples where the bulk analysis resulted in few single positive wells (> 15 % of all wells) is calculated according to the Spearmann and Kaerber formula.
Assuming the worst case, that these samples contain sufficient virus to infect all parallel cultures in the 1:3 higher concentrated dose from the final dilution of the sample incubated on the MTP. In this case only two reaction rates were reported. (mode E of calculation).

5.4 Calculation of the redUction factor

$$R = \log_{10} A_0 - \log_{10} A_n$$

R: reduction factor
A_0: viral titer / mL in the Load sample
A_n: viral titer / mL in the process samples and the Hold samples

Calculation of the standard deviation:

$$s_{eR} = \sqrt{s_{eA_0^2} + s_{eA_n^2}}$$

S_{eAo} standard deviation load sample
S_{eAn} standard deviation process samples and the hold samples

5.5 Calculation of clearance factor

$$R = \log_{10} A_0 - \log_{10} A_n$$

R: clearance factor
A_0: total virus load in the virus stock solution
A_n: total virus load in the process samples

Calculation of the standard deviation:

$$s_{eR} = \sqrt{s_{eA_0^2} + s_{eA_n^2}}$$

s_{eA0} standard deviation virus stock solution
s_{eAn} standard deviation process samples

6 Equipment and Material

Biochemicals/Buffers:	Supplier
o DMEM medium	Sigma D5796 Sigma P0781 PM A15-
o Penicillin-Streptomycin-solution	649 Sigma T4049
o Fetal calf serum	
o Trypsin/EDTA	

Disposables:	Supplier
o Pipettes (10 mL, 25 mL, and 50 mL)	Greiner 607 107 / 760 180 / 768 180
o Tubes (15 mL, 30 mL, and 50 mL)	Greiner 188 271/ 201 170 / 227 261
o Pipette tips, 200 µL	Greiner 702 556
o Pipette tips, 1000 µL	Greiner 702 561
o Pipette tips, 5000 µL	Eppendorf 022492080
o Microtiter plates, U-Form	Greiner 650 160
o Microtiter plates, F-Form	Greiner 655 182
o Lids for microtiter plates	Greiner 656 161
o Measuring Cylinders (different sizes)	Nalgene
o Buffer storage bottles (different sizes)	Nalgene / Schott
o Conical flasks (different sizes)	Schott
o Beakers (different sizes)	Schott / Brand
o Sidearm flasks (different sizes)	Schott
o Flasks 250 mL and 650 mL	Greiner 658 170 / 660 160
o Centrifuge beakers 200 mL	Nalgene
o Sterile filter (0.45 µm)	Millipore, Durapore

Instruments / equipment:	Supplier
o Multichannel pipette, adjustable	Brand, Transfer 8 and 12
o Adjustable pipette, 20-200 µL and 100-1000 µL	Brand, Transferpette
o Adjustable pipette, 500-5000 µL	Eppendorf
o Laminar-Flow-Bench	Heraeus Herasafe
o CO_2 -Incubator	Heraeus Heracell
o -75 to -85 °C freezer	Sanyo
o -15 to -35 °C freezer	Liebherr
o Refrigerator 2-8 °C	Dometic
o Microscope	Hund
o Centrifuge	Eppendorf 5810 R
o Cell counter	Coulter
o Photometer for cuvettes	Thermo Electron
o Submersable magnetic stirrer	Variomag
o Scale; 0.1 g to 620 g	Kern
o Scale; 1.0 g to 6000 g	Kern
o Temperature recorder (incl. probe)	Testo
o water bath	Julabo

7 Description of the Test Procedure

7.1 Pretestings

7.1.1 Toxicity assay

The toxicity assay was not performed. The data for Test Item A were already defined in study 2/06.166 (performed by NewLab BioQuality AG, Cologne) and was used in this study.

7.1.2 Interference assay

The interference assay was not performed. The data for Test Item A were already defined in study 2/06.166 (performed by NewLab BioQuality AG, Cologne) and was used in this study.

7.1.3 Recovery assay

10 mL of Test Item A were spiked with 1 mL virus stock solution. After mixture the spiked Test Item was incubated at room temperature for 2 hours. Subsequently, a sample was taken and analysed for the viral titer.

7.2 Description of the Test Procedure

The heat treatment was performed and documented by NewLab 's personnel in compliance with NewLab's Quality Assurance system.

Virus Spiking:
30.0 mL of Test Item 1 were spiked with 3.0 mL virus stock solution. After mixture a sample was taken and analysed for the viral titer by endpoint titration (load). As a second sample 7 mL were removed and kept at room temperature. The withdrawal of hold samples from this aliquot is described below.

Heat Treatment:
The spiked Test Item was placed into a water bath tempered to 8.0 ± 2.0 °C. For control of the water bath, one temperature probe (tp1) was placed into the water bath. Another temperature probe (tp2) was placed into a reference vial containing 30 mL of Test Item 1 to record the temperature throughout the whole process time.

The water bath was heated to 80 ± 2 °C within 103 minutes. Ramp samples were taken from the inactivation assay at 40 ± 2 °C (only for run 1), 50 ± 2 °C (only for run 1), and 60 ± 2 °C (for run 1 and 2), immediately cooled down in an ice bath and analysed for the viral titer by endpoint titration. In addition, the 60 °C sample was also analysed in large volume plating (bulk analysis).

A sample was taken 1 minute after reaching of 80 °C (for run 1 and 2), cooled down in an ice bath and analysed for the viral titer by endpoint titration and large volume plating (bulk analysis).

Withdrawal of hold samples:
From the 7 mL aliquot (see above) the following hold samples were withdrawn for analysis of the virus titer:

1. at the latest 2 minutes after withdrawal of the ramp sample 40 °C (see above) [1, 3]
2. at the latest 2 minutes after withdrawal of the ramp sample 50 °C (see above) [1, 3]
3. at the latest 2 minutes after withdrawal of the ramp sample 60 °C (see above) [2, 4]
4. at the latest 2 minutes after withdrawal of the 80 °C sample (see above) [2, 4]
5. after a duration of 11 hours calculated according to t_{0min} (see above) [2, 3]

[1]: performance of endpoint titration
[2]: in addition, performance of large volume plating (bulk analysis)
[3]: only for run 1
[4]: for run 1 and 2

The room temperature was between 19.4 and 22.1 °C.

8 Results

8.1 Toxicity assay

At a final dilution of 1:180 Test Item A was not toxic for KL-2 cells (data not shown here due to results of study 2/06.166).

8.2 Interference assay

At a final dilution of 1:180 the Test Item A did not interfere with the viral titer of BVDV (data not shown here due to results of study 2/06.166).

8.3 Recovery assay

In order to determine any immediate inactivation and/or neutralisation of the test virus by the Test Item A itself the process intermediate before heat treatment was spiked with BVDV. The viral titer after 2 hours incubation in Test Item A was compared to the viral titer of the virus stock solution.
Detailed data on the recovery of BVDV are given in tables 01 and in the appendix to this report.

8.4 Process validation

The capacity of heat treatment in the manufacturing process of Lipoprotein to inactivate virus was validated. For this purpose, the corresponding process intermediate was spiked with high-titer preparations of Bovine Viral Diarrhoea Virus (BVDV). The spiked Test Item were subjected to the inactivation procedure. Samples were withdrawn before, during and at the end of the treatment. Additionally, samples were withdrawn from an aliquot of the spiked Test Item which was stored at room temperature.
All samples taken were analysed for the viral titer by endpoint titration. In addition, several samples were also analysed by bulk analysis (large volume plating).

Process samples taken during heat treatment were diluted with cell culture medium prior to titration and bulk analysis. Titration of samples were performed at final dilutions that were neither cytotoxic nor did interfere with the viral titer.
Detailed data on the inactivation of BVDV are given in tables 02 and in the appendix to this report.
The following table summarizes the logio reduction factors determined for both assays mentioned above:
Table 2: Summary of the achieved $\log_{10}$ reduction factors

	Heat Treatment (80 °C after 103 min.)	Room temperature after 115 min. (1) / 111 min. (2)	Reduction induced by Heat Treatment only
	A *	B **	C ***
Maximum	4.10	1.47	2.65
Minimum	3.88	1.23	2.63

(1): for run 1
(2). for run 2

* Influence of the spiked Test Item subjected to Heat treatment
** Influence of the spiked Test Item at room temperature
*** Influence of the Heat treatment without any influence of the Test Item (C = A - B)

BVDV was considerably inactivated by the Test Item itself.
The reduction factor determined at the end of the heat treatment is the result of both effects the inactivation by heat and the inactivation by the Test Item itself.

9 Information on archival records

9.1 Archived by the Test Facility

The following records related to this study were archived at NewLab BioQuality AG Cologne according to the GLP principles:

- The study plan
- Raw data specific to this study
- All notes, measurement data and observations
- Reports of the Quality Assurance of study-based inspection and audits
- The Final Report
- Other raw data not specific but relevant to this study
  - Documents on test equipments - Standard Operating Procedures
  - Keeping and cultivation of the Test System

9.2 Archived by the Sponsor

Standard Operating Procedures of the Sponsor, laboratory documentation performed at NewLab, information on the Test Items as well as retention samples will be archived by the Sponsor.

10 Bibliography

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3. Note for Guidance on Quality of Biotechnological Products: Viral Safety Evaluation of Biotechnology Products derived from Cell Lines of Human or Animal Origin" (CHMP/ICH Q5A/295/95, Final Version, October 1997)
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11 Appendix

Recovery assay
BVDV

Virus	Label	Sample	Highest dilution of the Test Item inducing infection of all cultures	Observed reaction rates								log_{10} $TCID_{50}$/mL	Total volume (mL)	log_{10} total virus load	log_{10} clearence factor
				P1	P2	P3	P4	P5	P6	P7	sum	95% confidence limit		95% confidence limit	95% confidence limit
BVDV	R2B1	Virus stock solution	1.620.000	1,00	0,50	0,13	0,13	0,00	0,00	0,00	1,75	**7,51** ± 0,25	1,00	**7,51** ± 0,25	- -
	R2B2	Spiked Test Item (after 2 hours of incubation)	5.400	1,00	0,38	0,50	0,00	0,00	0,00	0,00	1,88	**5,09** ± 0,25	10,00	**6,09** ± 0,25	**1,42** ± 0,35

Factor of dilution: 3
Number of tested cultures per dilution: 8

Heat treatment
BVDV

run / date	Label	Sample	Mode of calculation	Highest dilution of the sample inducing infection of all cultures	Observed reaction rates								log_{10} TCID$_{50}$/mL	De-tection limit log_{10}	log_{10} reduction factor	Total volume (mL)
					P1	P2	P3	P4	P5	P6	P7	sum	95% confidence limit		95% confidence limit	
	1B10	Stock	A	1.620.000	1,00	0,88	0,13	0,13	0,00	0,00	0,00	2,13	**7,68** ± 0,21	4,57	- -	3,00
run 1 23.05.07	1B11	Load	A	145.800	1,00	0,75	0,50	0,25	0,00	0,00		2,50	**6,82** ± 0,29	2,57	- -	33,00
	1B12	40 °C ramp up	A	16.200	1,00	0,63	0,13	0,13	0,13	0,00	0,00	2,00	**5,62** ± 0,27	2,57	**1,19** ± 0,39	33,00
	1B13	50 °C ramp up	A	5.400	1,00	0,38	0,25	0,13	0,00	0,00	0,00	1,75	**5,03** ± 0,26	2,57	**1,79** ± 0,39	33,00
	1B14	60 °C ramp up	B	*80*	1,00	0,50	0,50	0,00	0,00	0,00	0,00	2,00	**3,32** ± 0,25	0,27	**3,50** ± 0,38	33,00
	1B15	80 °C target temperature	E	*60*	1,00	0,38						1,38	**2,72** ± 0,18	0,27	**4,10** ± 0,33	33,00
	1B16	Hold sample 40 °C	A	16.200	1,00	0,88	0,25	0,25	0,00	0,00	0,00	2,38	**5,80** ± 0,25	2,57	**1,01** ± 0,38	33,00
	1B17	Hold sample 50 °C	A	16.200	1,00	0,88	0,75	0,13	0,13	0,00	0,00	2,88	**6,04** ± 0,26	2,57	**0,78** ± 0,39	33,00
	1B18	Hold sample 60 °C	A	19.440	1,00	0,50	0,13	0,00	0,00	0,00	0,00	1,63	**5,53** ± 0,22	0,27	**1,29** ± 0,36	33,00
	1B19	Hold sample 80 °C	A	6.480	1,00	0,75	0,38	0,13	0,00	0,00	0,00	2,25	**5,35** ± 0,26	0,27	**1,47** ± 0,39	33,00
	1B20	Final Hold sample	B	*80*	1,00	0,50	0,38	0,25	0,00	0,00	0,00	2,13	**3,38** ± 0,30	0,27	**3,44** ± 0,41	33,00
run 2 23.05.07	2B11	Load	A	145.800	1,00	0,63	0,13	0,13	0,13	0,00		2,00	**6,58** ± 0,27	2,57	- -	33,00
	2B14	60 °C ramp up	B	*80*	1,00	0,00	0,13	0,00	0,00	0,00	0,00	1,13	**2,90** ± 0,12	0,27	**3,68** ± 0,30	33,00
	2B15	80 °C target temperature	E	*60*	1,00	0,33						1,33	**2,70** ± 0,17	0,27	**3,88** ± 0,32	33,00
	2B18	Hold sample 60 °C	A	6.480	1,00	0,75	0,75	0,25	0,00	0,00	0,00	2,75	**5,58** ± 0,27	0,27	**0,99** ± 0,38	33,00
	2B19	Hold sample 80 °C	A	6.480	1,00	0,75	0,38	0,13	0,00	0,00	0,00	2,25	**5,35** ± 0,26	0,27	**1,23** ± 0,38	33,00

Factor of dilution: 3
Number of tested cultures per dilution: 8