For Research Use Only December 17, 2012 Ver. 4

[Human Nesfatin-1 ELISA Kit]

(Code No.:AKHNF1-050)

Please, read this instruction carefully before use.

This kit is manufactured by Shibayagi Co., Ltd.
Use only the current version of Instruction Manual enclosed with the kit! For the detailed assay procedure, refer to Key points for ELISA by movie on our website:
http://www.shibayagi.co.jp/index-E.htm

1. Intended use

Human Nesfatin-1 ELISA Kit is a sandwich ELISA system for quantitative measurement of human Nesfatin-1. This is intended for research use only.

2. Storage and expiration

When the complete kit is stored at 2-8°C, the kit is stable until the expiration date shown on the label on the box. Opened reagents should be used as soon as possible to avoid loss in optimal assay performance caused by storage environment.

3. Introduction

Nesfatin-1 is a satiety molecule found in hypothalamic nuclei which regulate appetite. Its structure is corresponding to N-terminal 1-82 peptide sequence of a protein known as NEFA/nucleobindin2 (NUCB2). It is also found in rat cerebrospinal fluid and in peripheral tissues. Intra-cerebroventricular or intra-peritoneal administration of NUCB2 inhibits appetite in dose-dependent manner. Conversion of NUCB2 to nesfatn-1 is thought to be essential for food intake inhibition. Fasting decreases nesfatin-1 expression in paraventricular nucleus. Nesfatin-1 decreases body weight after chronic intra-cerebroventricular administration.

The anorexic action of nesfatin-1 is independent from leptin system. Its action has been suggested to be related to melanocortin signaling and to oxytocinergic signaling. Rat and human nesfatin-1s show 87.4% sequence homology. The active site of nesfatin-1 seems to be located in the middle segment. This segment has homology to α-MSH and agouti-related peptide (AgRP), and this sequence is thought to exert its action.

4. Assay principle

In Shibayagi's Human Nesfatin-1 ELISA Kit, standards or samples are incubated in polyclonal antibody-coated wells to capture Nesfatin-1. After 2 hours incubation and washing, biotin-conjugated anti-Nesfatin-1 antibody is added and incubated further for 2 hours to bind with captured Nesfatin-1. After washing, HRP- (horse radish peroxidase) conjugated avidin is added, and incubated for 30 minutes. After washing, bound HRP-conjugated avidin is reacted with a chromogenic substrate reagent (TMB) for 30 minutes, and reaction is stopped by addition of acidic solution, and absorbance of yellow product is measured spectrophotometrically at 450 nm. The absorbance is nearly proportional to Nesfatin-1 concentration. The standard curve is prepared by plotting absorbance against standard Nesfatin-1 concentrations. Nesfatin-1 concentrations in unknown samples are determined using this standard curve.

5. Precautions

- For professional use only, beginners are advised to use this kit under the guidance of experienced person. In manual operation, proficiency in pipetting technique is recommended.
- Use clean laboratory glassware.
- Avoid contact with the acidic Reaction stopper solution and Chromogenic substrate solution containing hydrogen peroxide and tetramethylbenzidine (TMB). Wear gloves and eye and clothing protection when handling these reagents.
- Be careful not to allow the reagent solutions of the kit to touch the skin, eyes and mucus membranes. Especially be careful for the reaction stopper because it is 1M sulfuric acid. The reaction stopper and the substrate solution may cause skin/eyes irritation. In case of contact with these wash skin/eyes thoroughly with water and seek medical attention, when necessary.
- Do not drink, eat or smoke in the areas where assays are carried out.

● In treating assay samples of animal origin, be careful for possible biohazards.
●This kit contains components of animal origin. These materials should be handled as potentially infectious.
●Unused samples and used tips should be rinsed in 1% formalin, 2% glutal aldehyde, or more than 0.1% sodium hypochlorite solution for more than 1 hour, or be treated by an autoclave before disposal.
●Dispose consumable materials and unused contents in accordance with applicable egional/national regulatory requirements.
●The materials must not be pipetted by mouth.
●In order to avoid dryness of wells, contamination of foreign substances and evaporation of dispensed reagents, never forget to cover the well plate with a plate seal supplied, during incubation.
●ELISA can be easily affected by your laboratory environment. Room temperature should be at 20-25°C strictly. Avoid airstream velocity over 0.4 m/sec. ① (including wind from air conditioner), and humidity less than 30%. ①For airstream, refer to [Assay circumstance] on our web site.

6. Reagents supplied

Components	State	Amount
(A) Anti-Nesfatin-1-coated plate	Ready for use.	96 wells/1 plate
(B) Standard human Nesfatin-1 (300 ng/ml) (derived from synthesis peptide)	Concentrated. Use after dilution.	200 µl/1 vial
(C) Buffer solution	Ready for use.	60 ml/1 bottle
(D) Biotin-conjugated anti-Nesfatin-1 antibody	Concentrated. Use after dilution.	200 µl/1 vial
(E) Peroxidase-conjugated streptavidin	Concentrated. Use after dilution.	200 µl/1 vial
(F) Chromogenic substrate reagent (TMB)	Ready for use.	12 ml/1 bottle
(H) Reaction stopper (1M H_2SO_4)Be careful!	Ready for use.	12 ml/1 bottle
(Ⅰ) Concentrated washing buffer (10x)	Concentrated. Use after dilution.	100 ml/1 bottle
Plate seal	−	4 sheets
Instruction Manual	−	1 copy

7. Equipments required but not supplied ☐Use as a check box
☐Purified water (distilled water)
☐Test tubes for preparation of standard solution series.
☐Glassware for dilution of washing buffer (a graduated cylinder, a bottle)
☐Pipettes (disposable tip type). One should be able to deliver 10 µl precisely, and another for 50-500 µl.
☐Syringe-type repeating dispenser like Eppendorf multipette plus which can dispense 100 µl.
☐Paper towel to remove washing buffer remaining in wells.
☐A vortex-type mixer.
☐A shaker for 96 well-plate (600-1,200rpm)
☐An automatic washer for 96 well-plate (if available), or a wash bottle with a jet nozzle. (refer to our web movie [Washing of microplate]).
☐A 96 well-plate reader (450nm ±10nm, 620nm: 600-650nm)
☐Software for data analysis, if available. Shibayagi is proposing the use of assay results calculation template for EXCEL. Please check our website (http://www.shibayagi.co.jp/en/tech_003.html).

8. Preparation of reagents
◆Bring all reagents of the kit to room temperature (20-25 °C) before use.
◆Prepare reagent solutions in appropriate volume for your assay. Do not store the diluted reagents.

【Concentrated reagents】

[(B) Standard human Nesfatin-1]

Below is an example of preparing each standard solution.

Volume of standard solution	Buffer solution	Concentration(ng/ml)
Original solution 50 µl	450 µl	30
30 ng/ml solution 250 µl	250 µl	15
15 ng/ml solution 250 µl	250 µl	7.5
7.5 ng/ml solution 250 µl	250 µl	3.75
3.75 ng/ml solution 250 µl	375 µl	1.50
1.5 ng/ml solution 250 µl	375 µl	0.60
0.60 ng/ml solution 250 µl	375 µl	0.24
Blank	250 µl	0

[(D) Biotin-conjugated anti-Nesfatin-1 antibody]

Prepare working solution by dilution of (D) with the buffer solution (C) to 1:100.

[(E) Proxidase-conjugated streptavidin]

Prepare working solution by dilution of (E) with the buffer solution (C) to 1:100.

[(I) Concentrated washing buffer (10x)]

Dilute 1 volume of the concentrated washing buffer (10x) to 10 volume with deionized water to prepare working solution. Example: 100 ml of concentrated washing buffer (10x) and 900ml of dionized water.

【Storage and stability】

[(A) Anti-Nesfatin-1-coated plate]

If seal is not removed, put the strip back in a plastic bag with zip-seal originally used for well-plate container and store at 2-8 °C. The strip will be stable until expiration date.

[(B) Standard human Nesfatin-1]

Standard solutions prepared above should be used as soon as possible, and should not be stored. Dispose remaining prepared solution.

[(C) Buffer solution] & [(F) Chromogenic substrate reagent]

Use only volume you need for your assay. Remaining reagents should be stored at 2-8 °C fastening the cap tightly. It maintains stability until expiration date. Once opened, we recommend using as soon as possible to avoid influence by environmental condition.

[(D) Biotin-conjugated anti-Nesfatin-1 antibody] & [(E) Proxidase-conjugated streptavidin]

Unused working solution (already diluted) should be disposed.

The rest of the undiluted solution: if stored tightly closed at 2-8 °C, it is stable until expiration date.

[(H) Reaction stopper (1 M H_2SO_4)]

Close the stopper tightly and store at 2-8 ° C. It maintains stability until expiration date.

[(I) Concentrated washing buffer (10x)]

The rest of undiluted buffer: if stored tightly closed at 2-8 °C, it is stable until expiration date.

Dispose any unused diluted buffer.

9. Technical tips

- In manual operation, proficiency in pipetting technique is recommended.
- The reagents are prepared to give accurate results only when used in combination within the same box. Therefore, do not combine the reagents from kits with different lot numbers. Even if the lot number is the same, it is best not to mix the reagents with those that have been preserved for some period.
- Be careful to avoid any contamination of assay samples and reagents. We recommend the use of disposal pipette tips, and 1 tip for 1 well.
- Optimally, the reagent solutions of the kit should be used immediately after reconstitution. Otherwise, store them in a dark place at 2-8 °C.
- Time the reaction from the pipetting of the reagent to the first well.
- Prepare a standard curve for each assay.
- Dilution of the assay sample must be carried out using the buffer solution provided in the kit.
- The chromogenic substrate (TMB) solution should be almost colorless before use. It turns blue during reaction, and gives yellowish color after addition of reaction stopper. Greenish color means incomplete mixing.
- To avoid denaturation of the coated antibody, do not let the plate go dry.
- As the antibody-coated plate is module type of 8wells x 12 strips, each strip can be separated by cutting the cover sheet with a knife and used independently.
- When ELISA has to be done under the airstream velocity over 0.4 m/sec. and the humidity less than 30%, seal the well plate with a plate seal and place the well plate in an incubator or a styrofoam box in each step of incubation. For more details, watch our web movie [Assay circumstance].

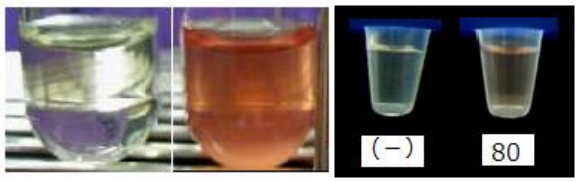
10. Preparation of samples

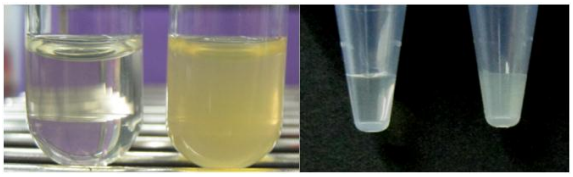
This kit is intended to measure Nesfatin-1 in human serum or plasma. The necessary sample volume for the standard procedure is 20 µl.

After collection of samples, store in ice, and centrifuge them, take out serum or plasma out of it and assay immediately or store below –35 °C until assay. Before starting assay, stir thawed samples sufficiently. Do not repeat freeze-and-thaw cycles.

Hemolytic and hyperlipemic serum samples are not suitable.

* To avoid influence of blood (high lipid or hemolysis, etc.), if your original samples have heavy chyle or hemolysis as the pictures below, do not use them for assay. Abnormal value might be obtained with hemolysis above 80mg/dL with this kit.





Normal | Hemolysis 80mg/dL | normal | Hemolysis 80mg/dL | Normal | Chyle Highly lipid sample | Normal | Chyle Highly lipid sample

If presence of interfering substance is suspected, examine by dilution test at more than 2 points. Dilution of a sample (5x as standard procedure) should be made in a test tube using buffer solution prior to adding them to wells. If Nesfatin-1 in blood is expected in low value, dilute the samples more than 2x. Turbid samples or those containing insoluble materials should be centrifuged before assay to remove any particulate matter.

Storage and stability

Nesfatin-1 in samples will be inactivated if stored at 2-8°C. If it is necessary to store samples in refrigerator (2-8°C), add aprotinin at final concentration of 100-500KIU/ml. (KIU: kallikrein inhibitor unit). If you have to store assay samples for a longer period, snap-freeze samples and keep them below –35°C. Defrosted samples should be mixed thoroughly for best results. Avoid repeated freeze-thaw cycles.

11. Assay procedure

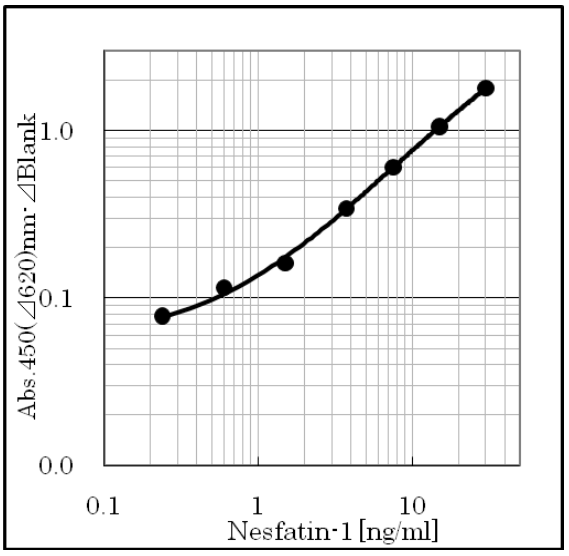
Remove the cover sheet of the antibody-coated plate after bringing up to room temperature.

(1) Wash the anti-Nesfatin-1-coated plate (A) by filling the wells with washing buffer and discard 4 times(*②), then strike the plate upside-down onto folded several sheets of paper towel to remove residual buffer in the wells.
(2) Pipette 100 µl of properly diluted samples to the designated sample wells.
(3) Pipette 100 µl of standard solution to the wells designated for standards.
(4) Shake the plate gently on a plate shaker(*③).
(5) Stick a plate seal (*④) on the plate and incubate for 2 hours at 20-25°C.
(6) Discard the reaction mixture and rinse wells as step (1).
(7) Pipette 100 µl of Biotin-conjugated anti-Nesfatin-1 antibody to all wells, and shake as step (4).
(8) Stick a plate seal (*④) on the plate and incubate the plate for 2 hours at 20-25°C.
(9) Discard the reaction mixture and rinse wells as step (1).
(10) Pipette 100 µl of Peroxidase-conjugated streptavidin to all wells, and shake as step (4).
(11) Stick a plate seal (*④) on the plate and incubate the plate for 30 minutes at 20-25°C.
(12) Discard the reaction mixture and rinse wells as step (1).
(13) Pipette 100 µl of Chromogenic substrate reagent to wells, and shake as step (4).
(14) Stick a plate seal (*④) on the plate and incubate the plate for 30 minutes at 20-25°C.
(15) Add 100 µl of the reaction stopper to all wells and shake as step (4).
(16) Measure the absorbance of each well at 450 nm (reference wavelength, 620*nm) using a plate reader within 30 minutes.

*Refer to the page 7-8 for notes of *②, *③ and *④.

12. Calculations

(1) Prepare a standard curve by plotting standard concentration on X-axis and absorbance on Y-axis. (Refer to our web site for more detailed explanation about standard curve. Shibayagi is offering a convenient Excel template. http://www.shibayagi.co.jp/en/tech_003.html)
(2) Using the standard curve, read the Nesfatin-1 concentration of a sample at its absorbance*, and multiply the assay value by dilution factor if the sample has been diluted. Though the assay range is wide enough, in case the absorbance of some samples is higher than that of the highest standard, please repeat the assay after proper dilution of samples with the buffer solution.
* We recommend the use of 3rd order regression curve for log-log plot, or 4 parameters method for log-normal plot in computer calculation.
Physiological or pathological situation of the subject should be judged comprehensively taking other examination results into consideration.



Human Nesfatin-1 assay standard curve (an example)
Absorbance may change due to assay environment.

13. Performance characteristics

● Assay range
The assay range of the kit is 0.24 ~ 30 ng/ml. (With 5x dilution, 1.2 ~ 150 ng/ml)

● Specificity

The antibodies used in this kit are specific to human Nesfatin-1.

Substances	Concentration	Cross reactivity (%)
Human Nesfatin-1	−	100
Rat Nesfatin-1	30 ng/ml	+
Human NUCB1-N77	20 µg/ml	-
Rat NUCB1-N77	20 µg/ml	-
Human CART	20 µg/ml	-
Human α-MSH	20 µg/ml	-
Human Leptin	20 µg/ml	-
Human Orexin	20 µg/ml	-
Human / Rat MCH	20 µg/ml	-
Human / Rat NPY	20 µg/ml	-

● Precision of assay

Within assay variation (2 samples, 5 replicates assay), Mean CV is 2.9 ~ 4.0%.

● Reproducibility

Between assay variation (3 samples, 4 days, duplicate assay), CV is 0.9 ~ 2.0%

● Recovery test

Standard Nesfatin-1 was added in 3 concentrations to 2 serum samples and were assayed. The recoveries were 94.7 ~ 103%

● Dilution test

Serum samples were serially diluted. The dilution curves showed linearity with $R^2 = 0.9997$.

14. Trouble shooting

● Low absorbance in all wells

Possible explanations:

1) The standard or samples might not be added.
2) Reagents necessary for coloration such as Biotin-conjugated anti-Nesfatin-1 antibody, Peroxidase-conjugated streptavidin, or Chromogenic substrate reagent might not be added.
3) Wrong reagents related to coloration might have been added. Wrong dilution of biotin-conjugated anti-Nesfatin-1 antibody or peroxidase-conjugated streptavidin.
4) Contamination of enzyme inhibitor(s).
5) Influence of the temperature under which the kits had been stored.
6) Excessive hard washing of the well plate.
7) Addition of chromogenic substrate reagent soon after taking out from a refrigerator might cause poor coloration owing to low temperature.

● Blank OD was higher that that of the lowest standard concentration (0.24 ng/ml).

Possible explanations:

Improper or inadequate washing. (Change washing frequency from 4 times to 5-8 times after the reaction with peroxidase-conjugated streptavidin.)

● High coefficient of variation (CV)

Possible explanation:

1) Improper or inadequate washing.
2) Improper mixing of standard or samples.
3) Pipetting at irregular intervals.

● Q-1: Can I divide the plate to use it for the other testing?

A-1: Yes, cut off the clear seal on the plate with cutter along strip. Put the residual plate, which is still the seal on, in a refrigerator soon

● Q-2: I found there contains liquid in 96 well-plate when I opened the box. What is it?

A-2: When we manufacture 96 well-plate, we insert preservation stabilizer in wells.

For detailed FAQS and explanations, refer to **"Trouble shooting and Important Points in Shibayagi's ELISA kits"** on our website (http://www.shibayagi.co.jp/en/tech_004.html).

15. References

*Indentification of Nesfatin-1 asa a satiety molecule in the hypothalamus.
Oh-I S, Shimizu H, Satou T, Okada S, Adachi S, Inoue K, Eguchi H, Yamamoto M, Imaki T, Hashimoto K, Tsuchiya T, Moden T, Horiguchi K, Yamada M, and Mori M.
Nature 443:709-712, 2006

*Peripheral Administration of Nesfatin-1 Reduces Food intake in Mice:
The Leptin-Independent Mechanism
Shimizu H, Oh-I S, Hashimoto K, Nakata M, Yamamoto S, Yoshida N, Eguchi H,.Kato I, Inoue K, Satoh T, Okada S, Yamada M, Yada T. and Mori M.
Endocrinology 150:662-671, 2009

Summary of assay procedure ☐ : Use as a check box

*First, read this instruction manual carefully and start your assay after confirmation of details.

For more details, watch our web movie [ELISA by MOVIE] on our website.

☐Bring the well-plate and all reagents back to 20-25°C for 2 hours.

☐Concentrated washing buffer must be diluted to 10 times by purified water that returned to 20-25°C.

☐Standard solution dilution example:

Concentration (ng/ml)	30	15	7.5	3.75	1.50	0.60	0.24	0
Std. solution (µl)→	Ori.Sol. 50	250*	250*	250*	250*	250*	250*	0
Buffer solution (µl)	450	250	250	250	375	375	375	250

*One rank higher standard.

☐Prepare the positive sample.

			Precautions & related info
☐	Anti-Nesfatin-1-coated plate		
☐	↓ Washing 4 times(*②)		*⑥
☐	Diluted samples, or Standards	100 µl	*⑦ [Handling of pipetting]
☐	↓ Shaking(*③), Incubation for 2 hours at 20-25°C. (Standing(*④))		*⑧ [Assay circumstance]
☐	Dilute Biotin-conjugated anti-Nesfatin-1 antibody (D) to 100x with buffer (C) returned to 20-25°C.		Dilute reagents during the first reaction.
☐	↓ Washing 4 times(*②)		*⑥
☐	Bioton-conjugated anti-Nesfatin-1 antibody	100 µl	*⑦ [Handling of pipetting]
☐	↓ Shaking(*③), Incubation for 2 hours at 20-25°C. (Standing(*④))		*⑧ [Assay circumstance]
☐	Dilute Peroxidase-conjugated streptavidin (E) to 100x with buffer (C) returned to 20-25°C.		Dilute reagents during the second reaction.
☐	↓ Washing 4 times(*②)		*⑥
☐	Peroxidase-conjugated streptavidin	100 µl	*⑦ [Handling of pipetting]
☐	↓ Shaking(*③), Incubation for 30 minutes at 20-25°C. (Standing(*④))		*⑧ [Assay circumstance]
☐	↓ Washing 4 times(*②)		*⑥
☐	Chromogenic substrate reagent (TMB)	100 µl	After dispense, the color turns to blue depending on the concentration.
☐	↓ Shaking(*③), Incubation for 30 minutes at 20-25°C. (Standing(*④))		*⑧ [Assay circumstance]
☐	Reaction stopper (1M H_2SO_4)	100 µl	After dispense, the color turns to yellow depending on the concentration.
☐	↓ Shaking(*③)		Immediately shake.
☐	Measurement of absorbance (450nm, Ref 620nm(*⑤))		Ref. wave cancels the dirt in the back of plate.

*②After dispensing wash buffer to wells, lightly shake the plate on your palm for 10 sec and remove the buffer. Guideline of washing volume: 300µl/well for an automatic washer and for

a pipette if the washing buffer is added by pipette. In case of washing by using 8 channel pipette, sometimes the back ground tends to be high. If so, change washing frequency from 4 times to 5-8 times at the constant stroke after the reaction with HRP conjugated streptavidin.
Standard of plate-washing pressure: 5-25ml/min. (Adjust it depending on the nozzle's diameter.) Refer to our web movie [Washing of microplate].

*③Guideline of shaking: 600-1,200rpm for 10 seconds x 3 times.

*④Seal the plate during the reaction after shaking. Peel off the protective paper from the seal and stick the seal on the plate. Do not reuse the plate seal used once.

*⑤600-650 nm can be used as reference wavelength.

*⑥After removal of wash buffer, immediately dispense the next reagent.

*⑦Refer to our web movie [Handling of pipetting].

*⑧Refer to our web movie [Assay circumstance].

Worksheet example

	Strip 1&2	Strip 3&4	Strip 5&6	Strip 7&8	Strip 9&10	Strip 11&12
A	30 ng/ml	Pos.Control.	Sample 8	Sample 16	Sample 24	Sample 32
B	15 ng/ml	Sample 1	Sample 9	Sample 17	Sample 25	Sample 33
C	7.5 ng/ml	Sample 2	Sample 10	Sample 18	Sample 26	Sample 34
D	3.75 ng/ml	Sample 3	Sample 11	Sample 19	Sample 27	Sample 35
E	1.50 ng/ml	Sample 4	Sample 12	Sample 20	Sample 28	Sample 36
F	0.6 ng/ml	Sample 5	Sample 13	Sample 21	Sample 29	Sample 37
G	0.24 ng/ml	Sample 6	Sample 14	Sample 22	Sample 30	Sample 38
H	0	Sample 7	Sample 15	Sample 23	Sample 31	Sample 39

Assay worksheet

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

[Storage condition] Store the kit at 2-8°C (Do not freeze).
[Term of validity] 6 months from production (Expiration date is indicated on the container.)

This kit is manufactured by **Shibayagi Co., Ltd.**
1062-1 Ishihara, Shibukawa, Gunma, Japan 377-0007
TEL.+81-279-25-0279, FAX.+81-279-23-0313
URL:http://www.shibayagi.co.jp/
E-mail: syc-info@shibayagi.co.jp

Distributed by: Xceltis
Xceltis GmbH, Pirnaer Str. 24
68309 Mannheim / Germany
Tel.: +49-(0)621-872096-0
Fax: +49-(0)621-872096-29
E-mail: info@xceltis.de
Internet: www.xceltis.de