SIGMA-ALDRICH®

SCAN BEC

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HybriScan®*D* Waste Water

Module 2

Microthrix parvicella

Rapid test system for the detection of *Microthrix parvicella* in waste water

Product-No.: 04447

Deutsche Version der Anleitung siehe unter www.sigma-aldrich.com/hybriscan

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Technical Service:

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Product Specifications

Cat. No.:	04447
Number of tests:	96 tests, incl. standard series
Storage:	4 – 8°C, 12 month
Test duration:	approx. 1-1.5 hours
Specificity:	*Microthrix parvicella*

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HybriScan®*D* Waste Water–Test Protocol

Working Principle

HybriScan™***D*** Waste Water is a new rapid molecular test system for the detection of bacteria in waste water. It consists of two modules; module 1- Waste Water Total Bacterial Count (Product No.: 78436) and module 2 Waste Water *Microthrix parvicella*.
The HybriScan® technology is based on the detection of target molecules from the microorganism of interest by means of specific capture and detection probes in a so-called sandwich hybridization. The target molecules of the microorganisms contained in the sample are captured in a specific microwell binding plate with the help of the capture probe. In addition to the capture probe, a detection probe is coupled to the target molecule. An enzyme is attached afterwards in a subsequent incubation step. After several washing steps, reaction with a colour substrate gives a blue colouration, which changes to yellow after the addition of a stop solution. The yellow colour enables highly sensitive photometric measurement at 450 nm. Comparison is made with the standard solutions contained in the test kit.
HybriScan®***D***–Waste Water Module 1 Total Bacterial Count is recommended to use only in combination with other Waste Water modules, like HybriScan™***D*** Waste Water Module 2 *Microthrix parvicella*. This is necessary for determination of the ratio of total bacterial amount and specific ascertained concentration of *Microthrix parvicella*. Furthermore environmental factors, like rain or variable influx can be considered *via* parallel using of both Waste Water modules. Module 2, Waste Water *Microthrix parvicella* can also be performed without module 1 as a single-mode-test.
In this manual test procedure is given including and excluding total bacterial count (Module 1)

Technical Notes

After starting the test procedure, perform the following steps without interruptions and within the given time limit.
For each sample use single-use pipette tip to avoid cross-contamination.
Close bottles immediately after use and store them at the temperatures specified on the label. Do not interchange caps and bottles.
Samples and standards should be tested together for more accurate results.
Do not mix or replace components from test kits of different charges.
Incubation at room temperature refers to a laboratory temperature of 20 to 25°C.
Do not use the test kit after the expiration date listed on the package.

Safety

All reagents contained in the test kit are for *in vitro* use only.
Test solution D contains formamide. Avoid contact with eyes, skin and the respiratory system. In event of contact with eyes or skin, rinse immediately with plenty of water. If the reagent is inhaled, immediately remove the individual to fresh air and seek medical attention. Stop solution H contains 1 N sulfuric acid. Avoid contact with eyes and skin. In the event of contact with eyes and skin rinse immediately with plenty of water.
Handling of the kit components and disposal of waste should be performed according to standard laboratory safety guidelines.

The handling of the kit components and the waste disposal should be performed according to the national safety guide lines.

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Reagents and Storage Conditions

The reagents contained in the test kit are sufficient for at least 96 tests, including 6 standard series. The kit components should be stored between +2 to +8°C as indicated on the labels. Do not freeze the test kit components!

Kit components:

1. Binding plate, ready to use, 96 wells	1
2. Standards [a] (white screw caps); **S1-MP**, **S2-MP**	0.2 mL each
3. Lysis Reagent A (red screw cap), ready to use	1.2 mL
4. Lysis Buffer B [a] (red cap), ready to use	4.5 mL
5. Lysis Buffer C [a] (red cap), ready to use	50 mL
6. Test Solution D-MP (yellow cap), ready to use	5.0 mL
7. Washing Solution E [b] (blue cap), ready to use	90 mL
8. Enzyme Solution F (green screw cap), dilute a suitable amount 1:100 with Washing Solution E before use	0.120 mL
9. Substrate Solution G [b] (green cap), ready to use	10 mL
10. Stop Solution H (green cap) 1 N sulfuric acid, ready to use	5 mL
11. Glass beads (colourless cap), sterile, ready to use	8 mL

[a] Components contain SDS, which precipitates at lower temperatures. Equilibrate to room temperature before use.
[b] Equilibrate to room temperature before use.

Additional equipment and materials (required, not supplied with kit)

- Centrifuge for microreaction tubes (1.5 and 2 mL)
- Thermoshaker for microreaction tubes and microwell plates
- Vacuum filtration unit
- 3 Pipettes (2–20 µL, 20–200 µL, 200-1000 µL) with corresponding tips; optional 8-channel pipette (20-200 µL)
- Microwell plate-photometer
- Microreaction tubes (2 mL), cultivation tubes (12 mL), reagent-reservoirs, membrane filter discs (0.45 µm)

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Test protocol- detection of *Microthrix parvicella* incl. parallel determination of Total bacterial Count

Additional material: HybriScanD Waste Water – Module 1 Total Bacterial Count (Product No.: 78436)

(1) Sample preparation

Transfer a 0.1 mL aliquot from the well mixed probe via pipette to a 2 mL microreaction tube. Centrifuge the samples for 2 minutes at maximum speed of 13,000 rpm. Remove the supernatant carefully with a pipette.

Note:

Avoid strong shaking after centrifugation to avoid resuspending the bacteria pellet. Centrifuge a second time if necessary.

(2) Cell lysis

Add 40 µL of **Lysis Buffer B** (bottle with red cap) to the cell pellet and 10 µL of **Lysis Reagent A*** (microreaction tube with red screw cap), add two spatula-tip amount of glass beads, mix well and incubate for 15 minutes at 37°C in a thermoshaker. Next, add 450 µL of **Lysis Buffer C** (bottle with red cap). Incubate again for 15 minutes at 37°C with shaking at 1,400 rpm in the thermoshaker. Centrifuge the samples for 10 minutes at 13,000 rpm using a microcentrifuge. Use 10 µL of this supernatant in protocol step 3 (hybridization).

***Note:** In the case of a large number of samples prepare a Master Mix of **Lysis Reagent A** and **Lysis Buffer B** before use. Pipette 50 µL of the Master Mix to each cell pellet.

Preparation of subsequent steps:

Change the top of the thermoshaker and fix the manifold for microwell plates. Set the temperature to 50°C and shaking speed to 500 rpm. To each well of standards **S1-TBC (A1)** and **S2 TBC (B1)** and samples add 45 µL of **Test Solution D-TBC** (bottle with yellow cap). To each well of standards **S1-MP (A2)** and **S2 MP (B2)** and samples add 45 µL of **Test Solution D-MP** (bottle with yellow cap). For parallel determination: see pipette scheme below!

Incubate the plate at 50°C for 5 minutes in the thermoshaker.

Pipette Scheme-Single assay:

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1-TBC	S1-MP										
B	S2-TBC	S2-MP										
C	TBC 1	MP 1										
D	TBC 2	MP 2										
E	TBC 3	MP 3										
F	TBC 4	MP 4										
G	TBC 5	MP 5										
H	TBC 6	MP 6										

TBC: Total Bacterial Count; MP: *Microthrix parvicella*
S: standards for Total Bacterial Count bzw. *Microthrix parvicella*

Analysis-data for every single probe consists of measured values for „MP"- and „TBC" (e.g. probe 1: MP1/TBC1)

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Pipette Scheme-Parallel determination:

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1-TBC	S1-TBC	S1-MP	S1-MP								
B	S2-TBC	S2-TBC	S2-MP	S2-MP								
C	TBC 1	TBC 1	MP 1	MP 1								
D	TBC 2	TBC 2	MP 2	MP 2								
E	TBC 3	TBC 3	MP 3	MP 3								
F	TBC 4	TBC 4	MP 4	MP 4								
G	TBC 5	TBC 5	MP 5	MP 5								
H	TBC 6	TBC 6	MP 6	MP 6								

TBC: Total Bacterial Count

MP: *Microthrix parvicella*

S: standards forTotal Bacterial Count bzw. *Microthrix parvicella*

Analysis-data for every single probe consists of each with two measured values for „MP"- and „TBC" (e.g. probe 1: (MP1+MP1)/(TBC1+ TBC 1))

(3) Hybridization and coupling to the binding plate

Add 10 µL of **Standard S1-TBC** into the well A1 and **Standard S2-TBC** into well B1; 10 µL of **Standard S1-MP** into A2 and **Standard S2-MP** into B2. Apply 10 µL of each sample (supernatant from step 2) into the respective well position. Cover the microwell plate with a lid and incubate it in the thermoshaker for 20 minutes at 50°C and 500 rpm.

For parallel determination: see pipette scheme above!

Note:

When adding the standards and samples, to avoid cooling do not remove the microwell plate from the thermoshaker.

The supernatant from step 2 can be stored at -20°C for future use.

Note:

Unused stripes of the plate should be stored in the sealed original packing at 4 to 8 °C.

Preparation for subsequent steps:

The **Enzyme Solution F-Washing Solution E** 1:100 dilution must be prepared immediately before use. It cannot be stored. Prepare only the amount needed for the test, e. g. for 16 reactions combine 1700 µL **Washing Solution E** and 17 µL **Enzyme Solution F**.

Note:

Briefly spin down enzyme solution F prior use to collect the liquid at the bottom of the tube.

(4) Enzymatic reaction

Discard the liquid from each well by inverting and gently tapping of the plate on an absorbent layer. Set the temperature to 25°C. Add 200µL **Washing Solution E** (bottle with blue cap) and incubate for 2 minutes at room temperature. Discard the liquid. Pipette 100 µl of the diluted **Enzyme Solution F**, prepared as described above " preparation of subsequent steps", to each well. Cover the binding plate with a lid and incubate it in the thermoshaker for 10 minutes at 25°C and 500 rpm.

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(5) Washing

Discard the liquid from each well. Add 200 µL of **Washing Solution E** (bottle with blue cap) to each well and incubate the microplate (with lid) for 1 minute at 25°C and 500 rpm in the thermoshaker. Repeat washing each well once.

Preparation for subsequent steps:

Switch on the computer and the microplate reader.

(6) Substrate Reaction

After discarding the Washing Solution from the second wash step, add 100 µL of **Substrate Solution G** (bottle with green cap) to each well. Cover the microplate with a lid and incubate it in a thermoshaker for 5 minutes at 25°C and 500 rpm. Stop the reaction by adding 50 µL of **Stop Solution H** (bottle with green cap) to each well. The addition of acid creates a yellow colour change. Mix shortly (10 sec, 500 rpm) in the thermoshaker and remove air bubbles, if present.

Note:

For qualitative analysis results can be measured by visual inspection. Compared to the blanks (A1, B1), which should be colourless, a blue colour change indicates contamination of the sample.

(7) Signal read-out using VIS-photometer

Start the reader and open the photometer Software. Insert the microwell plate into the reader, with position A1 rear left. Start the measurement. The instrument measures the absorbance of any position at 450 nm.

(8) Data analysis with Microsoft Excel

Open Excel-Data sheet "Calculator Waste Water English"*. Transfer absorption-data into the grey highlighted fields, consistent to the positions of your pipette scheme. Excel will calculate and show the results of measurement in given tables:

1. Microthrix-concentrations are calculated based on the standards
2. Total bacterial Count-concentrations are calculated based on the standards
3. Ratio Microthrix: Total Bacterial Count

* Excel- Data sheet for Data analysis: http://www.sigma-aldrich/hybriscanwater

(9) Data interpretation

Excel calculates via absorption values of the samples and standards results of analysis, which presents the relative concentration of *M. parvicella* in a ratio to the Microthrix or Total Bacterial Count standards.

The relevant standards must be absolutely in the stated positions. Otherwise Excel will calculate wrong results.

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Short Protocol

1. Remove 0.1 mL of sample, centrifuge (13,000 rpm, 2 minutes) and discard the supernatant
2. Add 40 µL of **Lysis Buffer B** (red cap) to the pellet; add 10 µL of **Lysis Reagent A** (red screw cap); add two spatula of glass beads, mix and incubate for 15 minutes at 37°C in a thermoshaker
3. Add 450 µL of **Lysis Buffer C** (red cap) and incubate for 15 minutes at 37°C and 1,400 rpm in the thermoshaker
4. Centrifuge for 10 minutes at 13,000 rpm
5. Pipette 45 µL of **Test Solution D** (yellow cap) per sample (including the standards) into the wells of a microplate and pre-incubate for 5 minutes at 50°C and 500 rpm in the thermoshaker
6. Add 10 µL of the supernatant from step 4 to each well (A1, B1, A2, B2 are reserved for the respective standards); cover the microwell plate with a lid and incubate for 20 minutes at 50°C and 500 rpm in the thermoshaker
7. Discard all liquid and wash the plate with 200 µL **Washing Solution E** (blue cap), discard Washing Solution
8. Dilute a suitable amount of **Enzyme Solution F** (green screw cap) 1:100 with **Washing Solution E** (blue cap) and add 100 µL of the mixture to each well of the microplate; cover the plate with a lid and incubate for 10 minutes at 25°C and 500 rpm in the thermoshaker
9. Discard all liquid and add 200 µL of **Washing Solution E** (blue cap) to each well and incubate for 1 minute at room temperature and 500 rpm in the thermoshaker; repeat the washing step once
10. Discard all liquid and add 100 µL **Substrate Solution G** (green cap) per sample to the wells of the microplate; cover the plate with a lid and incubate for 5 minutes at 25°C and 500 rpm in the thermoshaker
11. Add 50 µL **Stop Solution H** (green cap) to each well
12. Place the microplate in a microplate reader and measure the optical density in each well at 450 nm; perform data analysis

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Test protocol- detection of *Microthrix parvicella* exclusive parallel determination of Total bacterial Count

(1) Sample preparation

Transfer a 0.1 mL aliquot from the well mixed probe via pipette to a 2 mL microreaction tube. Centrifuge the samples for 2 minutes at maximum speed of 13,000 rpm. Remove the supernatant carefully with a pipette.

Note:

Avoid strong shaking after centrifugation to avoid resuspending the bacteria pellet. Centrifuge a second time if necessary.

(2) Cell lysis

Add 40 µL of **Lysis Buffer B** (bottle with red cap) to the cell pellet and 10 µL of **Lysis Reagent A** (microreaction tube with red screw cap), add two spatula-tip amount of glass beads, mix well and incubate for 15 minutes at 37°C in a thermoshaker. Next, add 450 µL of **Lysis Buffer C** (bottle with red cap) are. Incubate again for 15 minutes at 37°C with shaking at 1,400 rpm in the thermoshaker. Centrifuge the samples for 10 minutes at 13,000 rpm using a microcentrifuge. Use 10 µL of this supernatant in protocol step 3 (hybridization).

Note: Add the **Lysis Reagent A** to the **Lysis Buffer B** before use.

Preparation of subsequent steps:

Change the top of the thermoshaker and fix the manifold for microwell plates. Set the temperature to 50°C and shaking speed to 500 rpm. To each well of standards **S1-MP (A1)** and **S2 MP (B1)** and samples add 45 µL of **Test Solution D-MP** (bottle with yellow cap). For parallel determination: see pipette scheme below!

Incubate the plate at 50°C for 5 minutes in the thermoshaker.

Pipette Scheme-Single assay:

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1-MP											
B	S2- MP											
C	MP 1											
D	MP 2											
E	MP 3											
F	MP 4											
G	MP 5											
H	MP 6											

MP: *Microthrix parvicella*; S: standards for *Microthrix parvicella*

Analysis-data for every single probe consists of measured values for „MP"- and „S-MP"

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Pipette Scheme-Parallel determination:

	1	2	3	4	5	6	7	8	9	10	11	12
A	**S1-MP**	**S1-MP**										
B	**S2- MP**	**S2- MP**										
C	MP 1	MP 1										
D	MP 2	MP 2										
E	MP 3	MP 3										
F	MP 4	MP 4										
G	MP 5	MP 5										
H	MP 6	MP 6										

MP: *Microthrix parvicella*

S: standards for *Microthrix parvicella*

Analysis-data for every single probe consists of measured average values for „MP"- and „S-MP"

(3) Hybridization and coupling to the binding plate

Add 10 µL of **Standard S1-MP** into A1 and **Standard S2-MP** into B1. Apply 10 µL of each sample (supernatant from step 2) into the respective well position. Cover the microwell plate with a lid and incubate it in the thermoshaker for 20 minutes at 50°C and 500 rpm.

For parallel determination: see pipette scheme above!

Note:

When adding the standards and samples, to avoid cooling do not remove the microwell plate from the thermoshaker.

The supernatant from step 2 can be stored at -20°C for future use.

Note:

Unused stripes of the plate should be stored in the sealed original packing at 4 to 8 °C.

Preparation for subsequent steps:

The **Enzyme Solution F-Washing Solution E** 1:100 dilution must be prepared immediately before use. It cannot be stored. Prepare only the amount needed for the test, e. g. for 16 reactions combine 1700 µL **Washing Solution E** and 17 µL **Enzyme Solution F**.

Note:

Briefly spin down enzyme solution F prior use to collect the liquid at the bottom of the tube.

(4) Enzymatic reaction

Discard the liquid from each well by inverting and gently tapping of the plate on an absorbent layer. Set the temperature to 25°C. Add 200µL **Washing Solution E** (bottle with blue cap) and incubate for 2 minutes at room temperature. Discard the liquid. Pipette 100 µl of the diluted **Enzyme Solution F**, prepared as described above " preparation of subsequent steps", to each well. Cover the binding plate with a lid and incubate it in the thermoshaker for 10 minutes at 25°C and 500 rpm.

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(5) Washing

Discard the liquid from each well. Add 200 µL of **Washing Solution E** (bottle with blue cap) to each well and incubate the microplate (with lid) for 1 minute at 25°C and 500 rpm in the thermoshaker. Repeat washing each well once.

Preparation for subsequent steps:

Switch on the computer and the microplate reader.

(6) Substrate Reaction

After discarding the Washing Solution from the second wash step, add 100 µL of **Substrate Solution G** (bottle with green cap) to each well. Cover the microplate with a lid and incubate it in a thermoshaker for 5 minutes at 25°C and 500 rpm. Stop the reaction by adding 50 µL of **Stop Solution H** (bottle with green cap) to each well. The addition of acid creates a yellow colour change. Mix shortly (10 sec, 500 rpm) in the thermoshaker and remove air bubbles, if present.

Note:

For qualitative analysis results can be measured by visual inspection. Compared to the blanks (A1, B1), which should be colourless, a blue colour change indicates contamination of the sample.

(7) Signal read-out using VIS-photometer

Start the reader and open the photometer Software. Insert the microwell plate into the reader, with position A1 rear left. Start the measurement. The instrument measures the absorbance of any position at 450 nm.

(8) Data analysis with Microsoft Excel

Open Excel-Data sheet "Software Waste Water English"*. Transfer absorption-data into the grey highlighted fields, consistent to the positions of your pipette scheme. Excel will calculate and show the results of measurement in given tables:

1. **Microthrix-concentrations are calculated based on the standards**
2. Total bacterial Count-concentrations are calculated based on the standards (not used)
3. Ratio Microthrix: Total Bacterial Count (not used)

* Excel- Data sheet for Data analysis: http://www.sigma-aldrich/hybriscanwater

(9) Data interpretation

Excel calculates via absorption values of the samples and standards results of analysis, which presents the relative concentration of *M. parvicella* in a ratio to the Microthrix standards.

The relevant standards must be absolutely in the stated positions. Otherwise Excel will calculate wrong results.

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Short Protocol

1. Remove 0.1 mL of sample, centrifuge (13,000 rpm, 2 minutes) and discard the supernatant
2. Add 40 µL of **Lysis Buffer B** (red cap) to the pellet; add 10 µL of **Lysis Reagent A** (red screw cap); add two spatula of glass beads, mix and incubate for 15 minutes at 37°C in a thermoshaker
3. Add 450 µL of **Lysis Buffer C** (red cap) and incubate for 15 minutes at 37°C and 1,400 rpm in the thermoshaker
4. Centrifuge for 10 minutes at 13,000 rpm
5. Pipette 45 µL of **Test Solution D** (yellow cap) per sample (including the standards) into the wells of a microplate and pre-incubate for 5 minutes at 50°C and 500 rpm in the thermoshaker
6. Add 10 µL of the supernatant from step 9 to each well (A1, B1, A2, B2 are reserved for the respective standards); cover the microwell plate with a lid and incubate for 20 minutes at 50°C and 500 rpm in the thermoshaker
7. Discard all liquid and wash the plate with 200 µL **Washing Solution E** (blue cap), discard Washing Solution
8. Dilute a suitable amount of **Enzyme Solution F** (green screw cap) 1:100 with **Washing Solution E** (blue cap) and add 100 µL of the mixture to each well of the microplate; cover the plate with a lid and incubate for 10 minutes at 25°C and 500 rpm in the thermoshaker
9. Discard all liquid and add 200 µL of **Washing Solution E** (blue cap) to each well and incubate for 1 minute at room temperature and 500 rpm in the thermoshaker; repeat the washing step once
10. Discard all liquid and add 100 µL **Substrate Solution G** (green cap) per sample to the wells of the microplate; cover the plate with a lid and incubate for 5 minutes at 25°C and 500 rpm in the thermoshaker
11. Add 50 µL **Stop Solution H** (green cap) to each well
12. Place the microplate in a microplate reader and measure the optical density in each well at 450 nm; perform data analysis