Instructions for Use

AMODIA

easyFlow® Bacteria

Molecular test system
for the detection of

RNA of organisms of the domain Bacteria

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REF:
AEF-B01

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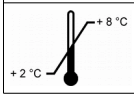
Inhaltsverzeichnis

Explanation of Symbols

Symbol	Explanation
	Expiry date
IVD	*In Vitro* Diagnostic Medical device
LOT	Batch code
REF:	Catalog number
	Storage conditions

Symbol	Explanation
	Consult Instructions for Use
	Consult attended documents
	Package size
	Manufacturer
	Only for evaluation purposes

Introduction

The easyFlow® detection method is based on the hybridization of ribosomal RNA with labeled probes. Several regions of the ribosomal RNA are characteristic for a species like a human fingerprint. In dead or not replicating cells the RNA is degraded quickly, thus only viable replicating cells are detected. For usage of the easyFlow® assay no amplification techniques (e.g. PCR) are necessary.

Description of the Test

The test-kit AMODIA easyFlow® Bacteria is intended for microbial quality control. It detects the RNA of viable and replicating bacteria (domain Bacteria) directly from an enrichment culture.
The easyFlow® Bacteria test-kit does not use amplification to detect bacteria. It consists of the moduls: a) hybridization of probes to 16S rRNA and b) detection of the formed complex on Lateral-Flow Dipsticks (LFDs).

Materials Supplied, Storage and Stability

Components	Cat.-No.	Content (50 tests)	Preparations	Storage	Stability / Shelf life
Hybridization					
Hybridization Buffer (transparent cap)	HBP01	1 vial a 1,8 ml	ready to use	room temperature	Until expiration date; 60 days after opening
Probe Mix (yellow cap)	PME01	1 vial a 300 µl	ready to use	4 - 8°C	Until expiration date; 60 days after opening
Detection					
Lateral Flow Dipsticks	LFD01	2 vials a 25 pcs.	ready to use	4 - 8°C	Until expiration date; 60 days after opening*
Chromatographic Buffer (blue cap)	ChB01	1 vial a 10 ml	ready to use	4 - 8°C	Until expiration date; 60 days after opening

* : LFD vials must be locked tightly! Storage with opened LFD vial reduces the stability of the LFDs.

Important note:
Expiration dates should not be exceeded.

Laboratory Instruments and Materials Required

- Thermal cycler with led heater
- Adjustable pipettes for 10 µl, 100 µl and 1.000 µl
- Rack made of plastic or aluminium, suitable for the PCR tubes
- Microwell plate
- Centrifuge for 1,5 ml reaction tubes
- PCR tubes compatible with the thermal cycler
- Sterile pipette tips with contamination protection (filter tips)

Warnings and Precautions

All reagents of this test-kit are strictly intended for the specified *in vitro* diagnostic use only.
Please adhere strictly to the sequence of processing steps provided by this protocol.
Store all reagents in the original vials at the temperatures indicated on the respective labels. Do not interchange kit components of different lots and assays. Do not use kit components beyond their expiration dates.
Stick to the safety rules for handling kit reagents and sample materials. Especially be aware of the following precautions:

- do not eat, drink or smoke
- wear safety clothes and gloves
- avoid contact with reagents and sample materials

Some reagents may contain preservation substances against microbial growth, so avoid contact with skin and / or mucous membranes.

Empy vials could be discarded with the normal laboratory waste.

Test Characteristics

Distributor AMODIA Bioservice GmbH
Order-No.: AEF-B01
Package size: 50 reactions
Delievery: from stock in Braunschweig, Germany
Storage: see „materials supplied, storage and stability“
Sample material: no manufacturer`s warranty!
Every sample material has to be evaluated by the operator!

	sample material
Required sample material:	1 ml liquid enrichment culture
Analytic specifity:	highly specific for: Bacteria coverage of 91 % in the domain bacteria

Test time and procedure:

Hybridization set-up	approx. 10 minutes
Hybridization reaction	approx. 35 minutes
Detection and read-out	approx. 15 minutes
Total test time +	approx. 1 hour

+ : with heating- and cooling rates of 1,5°C/s

Examples for Liquid Enrichment Cultures

It is necessary to perform an enrichment culture prior to the detection reaction. Procedures already validated should be used unchanged for the preparation of these enrichment cultures. This is why there are no reagents for this included in the kit.
The volume of the enrichment culture can be adapted. The following example demonstrates the required adjustments of the volumina.

	Steps to prepare the Enrichment Culture
1.	Pipet **1 g product** (~ 1 ml) into a sterile reaction tube. Use a fresh pipette tip or transfer pipette for each sample.
2.	Q.S. to **10 ml** with an appropriate **inactivation solution***.
3.	Mix samples for **at least 60 s** until a **homogeneous** solution has formed. Incubate the solution for **10 to 30 min** at room temperature.
	Alternative A: 100 ml enrichment culture
4.a	Pour **90 ml nutrient solution**** into a suitable flask.
5.a	Add **10 ml of the sample solution** and incubate for **14 – 24 hours at 30°C.**
	Alternative B: 10 ml enrichment culture (lower sensitivity possible!)
4.b	Pour **9 ml nutrient solution**** into a suitable flask (e.g. 50 ml Falcon tube).
5.b	Add **1 ml of the sample solution** and incubate for **14 – 24 hours at 30°C.**

*: solution that inactivates preservatives in the tested sample
**: see note

Note:
Nutrient solutions with ingredients for inactivation of preservatives (e.g. Eugon LT broth) should be used according to manufacturer`s specifications.

Assay Conditions

Important notes:
- **Open and close the reaction vials individually.**
- **Change gloves immediately, if contaminated.**
- **Open the reaction tubes carefully in order to avoid aerosols. If possible centrifuge for a few seconds.**
- **Hybridized products (RNA-probe conjugate) should be treated as an important source of potential contamination.**
- **Do not interchange components of different lots.**

1. Modul: Hybridization

Additional reagents to be supplied by user: **TE buffer** (10 mM Tris-HCl, 1 mM EDTA; pH 8), **lysozyme**

1.1 Pre-treatment

For RNA extraction centrifuge 1 ml enrichment culture for 10 min at 13`000g. The supernatant is discarded completely. The sediment is mixed with 100 µl TE buffer containing 1 mg/ml lysozyme. Incubate 37 °C for 10 min. Now the samples are ready for the hybridization reaction.

Warning:
It is necessary to remove as much culture medium as possible after centrifugation. Otherwise false-positive results may occur.

	Steps for RNA Extraction
1.	Prepare and label the required number of sterile 1,5 ml reaction tubes.
2.	For every sample transfer **1 ml enrichment culture** into single reaction tubes. For each tube use a new pipette tip. Close the tubes individually.
3.	Centrifuge the samples **at 13´000g for 10 min**.
4.	Remove the supernatant completely from the sediment. For each tube use a new pipette tip. Close the tubes individually.
5.	With a new filter tip for every sample pipet **100 µl TE buffer containing 1 mg/mL lysozyme** into the reaction tubes. **Suspend** the sediment in the buffer by **pipetting several times up and down** or **vortex** for **10 s**. Incubate at **37 °C for 10 min.** Open tubes **individually** und close them immediately after pipetting.

1.2 Preparation of Reaction Mixture

Into each sterile reaction tube (PCR-tube) pipet **30 µl hybridization buffer** and **5 µl probe mix**. Add **100 µl lysed** sample from 1.1.
For more than one test reaction the preparation of a mastermix is recommended. Therefor the volumes for a single reaction have to be multiplied with the factor of reactions (samples + controls + 1).

Hybridization Mixture		**Example: mastermix for 10 reactions**
Reagent	**Volume per reaction**	**Factor = 11**
Hybridization Buffer	**30,0 µl**	330,0 µl
Probe Mix	**5,0 µl**	55,0 µl
		pipet 35 µl into each of the 10 reaction tubes, discard the remaining mixture
Enrichment culture or colony in buffer	**100,0 µl**	add 100 µl **individually**

	Steps for Hybridization
1.	Prepare and label the required number of sterile PCR tubes. Process all pipetting steps without interruption. It is recommended to process a negative control in every test run.
2.	Prepare mastermix (see above).
3.	Pipet **35 µl mastermix** into the reaction tubes. **Close the reaction tubes.**
4.	Add **100 µl sample solution** from 1.1 to the reaction mixture. For each tube use a new pipette tip. Open tubes individually and close them immediately.
5.	Negative controls contain no further material.

1.3 Hybridization Reaction

Place the samples in the thermal cycler and start the hybridization program.

The assay was developed using a Primus thermal cycler from PEQLAB. If a different thermal cycler is used an adaption of the program may be necessary. If you have any questions, please contact the manufacturer of the test-kit.

Programming of the thermal cycler for the hybridization reaction

Set lid heating to 110°C.

Denaturation	**temperature***	5 min*	at 95°C
Annealing	**ramp****		0,1°C/s to 50°C
	temperature*	5 min*	at 50°C
Cooling	**temperature***	3 min*	at 20°C

*: The specified times given are the reaction times **without** the time required for cooling and heating!
: means: slow cooling from **95°C to 50°C, following a step at 50°C for 5 min.

The total duration time of the hybridization program is about 25 min.

2. Modul: Product Detection

Attention:
- Do not interchange components of different lots.
- **Hybridized RNA-probe conjugates** are an important **source of contamination** and should be pipetted and disposed with care.

Overview

Lateral-Flow Dipsticks (v. Fig. 1) are used for the detection. They consist of an area of sample application (purple), a membrane and an absorption area (white) With exception of the application area the whole strip is covered by a tape. The strip can be touched at the covered areas. Remarks should be made only on the tape above the absorption area.
Hybridization products are applied directly onto the Lateral-Flow Dipstick.

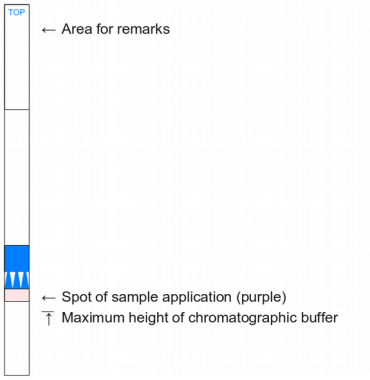


Fig. 1: Layout of the Lateral-Flow Dipsticks

2.1 Detection

	Steps for Detection
1.	Prepare the required number of LFDs and label them. Only touch the areas covered with tape and use the white area at the end of the LFD for labeling. Close the LFD vial tightly.
2.	Pipet **150 µl chromatographic buffer** into single vials or wells of a microplate for each reaction.
3.	Pipet **10 µl** of the hybridization reaction close to the edge of the tape marked with triangles onto the free membrane part of the LFD (v. Fig. 1). A bleeding of the liquid is normal.
4.	Dip the LFDs with the area without tape into the chromatographic buffer prepared in step 2. Allow an incubation of **20 min**. Make sure that the application area is discolored. The control line must be visible for interpretation of results. The lines are stable and can be read later.

2.2 Interpretation of Results

1.	**Two** lines are visible: test line and control line (v. Fig. 2) **Notes**: Even a faint test line has to be interpreted as positive. For a comparison the negative control should be considered. If necessary the test must be repeated for confirmation. Positive results may be visible even before the entire incubation time is over.	The detection of RNA of bacteria is **positive.**
2.	**Only one** test line is visible at the location of the control line. **Note** Do not interpret the results until the incubation time is over.	The detection of RNA of bacteria is **negative.**

The result of the test is only valid if the control line of every sample is visible.

The **negative control** for the complete test run has to be correct in order to validate the results. If the negative control shows a visible test line, the analysis of **all** samples tested in parallel must be repeated.

The dried test strips could be adhered to an evaluation sheet and stored.

The test-kit gives only a qualitative result. The intensity of the stained test line has no direct relation to the number of cells present in a positive sample.

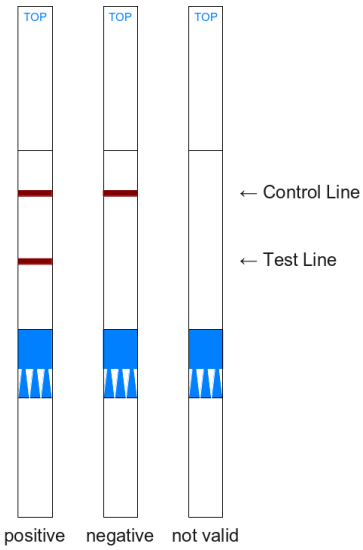


Fig. 2.: Interpretation of results

Method / Test Principle

1.) Sample Preparation

The preparation of an enrichment culture is necessary to ensure that enough RNA molecules for detection are available. Only replicating microorganisms produce RNA, so that only viable micro-organisms are detected. Nucleic acids (e.g. DNA) of non-replicating or dead cells are not detected. A centrifugation step concentrates the cells of microorganisms eventually present in the samples and removes residue of the enrichment culture. The sediment is suspended in TE buffer containing lysozyme to destroy the cell walls.

2.) Hybridization

A part of the suspended sample solution is mixed with the probe mix. During the following temperature controlled hybridization reaction the cells are lysed. Labelled probes hybridize to the released ribosomal RNA. This RNA-probe complex is detected directly on the LFD.

3.) Detection

The detection is performed using an immuno-chromatographic assay on a Lateral-Flow Dipstick. With this device the hybridization product is detected.
The hybridized complex of RNA and probe binds to an antibody, which is immobilised on gold particles. With the diffusion of the chromatographic buffer the complex also diffuses through the membrane. This membrane is prepared with two lines of different capture molecules. At the first line only gold particles are bound which are also linked to the hybridization product. This binding increases the local concentration of the gold thus forming a visible line. Gold conjugates without hybridization products diffuse further and bind at the second line. Here the line gets visible if the gold conjugate is still intact. This serves as a control for the correct performance of the Lateral-Flow Dipstick.

Trouble-Shooting

Problems	Possible cause	Recommendation
No growth in enrichment culture	a) Inappropriate growth temperature	a) Check optimal growth temperature
	b) Inappropriate nutrient solution	b) Use nutrient media (e.g. Caso) instead of selective media
	c) Inactivation solution does not work	c) Use freshly prepared inactivation solution or try different solution
No control line visible (upper line)	a) Chromatographic buffer wrong or disfunctional	a) Use new chromatographic buffer
	b) Test strips expired	b) Use new test strips
	c) Wrong storage of test strips	c) Store at 2 - 8°C
All samples and controls show a positive result.	a) Contamination of hybridization reaction	a) Clean bench and pipettes, check reagents.
	b) Chromatographic buffer is contaminated.	b) Use new chromatographic buffer
Positive control shows a negative signal	a) No or wrong hybridization buffer or probe mix added	a) Repeat hybridization, check reagents
	b) Hybridization failed	b) Check hybridization program
	c) Inhibition by tested product	c) Repeat test, use appropriate inactivation solution or dilution

Attachment

- Template LFD documentation
- Protocol at a glance