RiboFlow® *Cosmetics* Detection Kit

for the detection of microorganisms specified according to ISO 17516

Manual, Version 2, August 2015

Product number 51-420113

6 sets, 4 assays each

Store at: +2 to +25°C

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RiboFlow® *Cosmetics* Detection Kit

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1. General Information

1.1 Kit components and storage

Solution A, 2 ml
Solution B, 1 ml
Solution C, 2 ml
RiboFlow® *Cosmetics* Lateral Flow Assay devices, 6 sets, 4 test cassettes each (*E. coli, S. aureus, P. aeruginosa, C. albicans*)
Reaction tubes 2 ml, 8 pcs.
Manual

All kit components can be stored at +2 to +25°C.

1.2 Accessory materials and equipment

Required:
- Micropipettes
- Sterile pipette tips
- Incubator for enrichment cultures
- Suitable sample diluents and enrichment media (refer to section 2)
- Magnetic stirrer and magnetic stirring rods
- Microcentrifuge (relative centrifugal force at least 2000 × g), SY-LAB Geräte GmbH, product number 51-410000
- Mini-Incubator IL10 (SY-LAB Geräte GmbH, product number 51-410100 or VWR international, product number 390-0384)
- RiboFlow® manipulation plate, SY-LAB Geräte GmbH, product number 51-410110

Optional / recommended:
- BacTrac impedance analyser and suitable media (contact SY-LAB Geräte GmbH for further information)
- Non-selective medium such as Brain Heart Infusion Broth (BHI), and additional sterile 2 ml reaction tubes (for sub-cultivations of older cultures and/or cultivation + confirmation of suspicious single colonies from selective agar plates, if appropriate)

1.3 Product use / Scope

The RiboFlow® *Cosmetics* Detection Kit can be used to detect the four specified microorganisms according to ISO 17516, i. e. *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans* in cosmetics samples using

- Liquid cultures in enrichment media or reactive samples from impedance analysis, flow cytometry or ATP measurements
- Sub-cultures of enrichments or of single colonies
- single colonies from agar plates

This kit is not approved for clinical use. During performance of the test protocol, all due care and attention should be exercised in handling kit components (see chapter 1.4 “Safety information“).

1.4 Safety information

When handling with RiboFlow® kit components, please refer to the respective Material Safety Data Sheets. These are available on our website (www.sylab.com) for download by registered users (Microbiology, Service & Downloads / Molecular Microbiology section). Please observe general safety measures for handling chemicals. Never store kit components together with food. Always wear disposable gloves, protective goggles and suitable protective clothing when working with chemicals.

Caution: *Follow your national safety regulations for handling of microorganisms and take the appropriate measures to prevent infections. Inactivate contaminated material by disinfection and autoclaving.*

1.5 Product warranty and limitation of warranty

SY-LAB Geräte GmbH guarantees the performance of this product as described in chapter 1.7 “Specifications/Performance“ and for the intended use to the expiration date given on the label. The purchaser must determine the suitability of the product for its particular use and adjust reaction conditions if necessary. SY-LAB Geräte GmbH does not assume responsibility for any consequences or damage whatsoever resulting from use of this product. Should the product fail due to any reason other than misuse or incorrect storage, SY-LAB Geräte GmbH will replace it free of charge or refund the purchase price after written

agreement. We reserve the right to change this product anytime to enhance performance or design. Should there be any technical problems, please do not hesitate to contact us for quick and straightforward help.

1.6 Quality control

Quality and assay performance of this product are monitored for each lot following Standard Operating Procedures. Quality control certificates are available on our website (www.sylab.com) for download by registered users (Microbiology, Service & Downloads / Molecular Microbiology section).

1.7 Specifications/Performance

This test kit was developed to ensure detection of ≥ 1 cfu of a microorganism specified acc. to ISO 17516 in 1 g of product, provided that the protocols described in this manual are followed.
A consistent analytical limit of detection of 1.6×10^{10} copies of the respective target nucleic acid molecules is verified for each lot of RiboFlow® *Cosmetics* Detection Kit.

1.8 Customer service

For technical advice, please contact our customer service (E-mail: supportbio@sylab.com, phone: +43-2231-62252-0, fax: +43-2231-62193).
As our customer you are a valuable source of information concerning your special applications and requirements. Your feed-back, information and comments are very helpful for us, since we constantly seek to enhance our products. Please contact us if you have suggestions concerning our products.

1.9 Introduction

Routinely, the detection of the four microorganisms classified as undesirable according to ISO 17516 is achieved by using classical microbiological and biochemical methods, which are characterized by long duration and high costs. Thus, faster, reliable molecular biology-based methods are in great demand. A rapid method based on molecular biology considerably reduces time and costs, in addition often combined with enhanced specificity compared to microbiological/biochemical methods. The RiboFlow® *Cosmetics* Detection Kit was developed to enable highly specific yet very simple detection of the four specified microorganisms according to ISO 17516, which are potential human pathogens (*E. coli, S. aureus, P. aeruginosa* and *C. albicans*) within just a few minutes, with little effort and equipment.

1.10 Test principle

With RiboFlow® *Cosmetics*, ribosomal RNA sequences specific for *E. coli, S. aureus, P. aeruginosa* and *C. albicans* are detected by a proprietary nucleic acid hybridisation protocol in a simple lateral flow assay single test format within 30 minutes, using a crude cell extract from an enriched culture or a single colony. Tedious nucleic acid purification or enzymatic amplification of target sequence is not necessary.

2. Protocol

Note: This assay is intended for the analysis of enriched samples (conventional enrichments, enrichments from impedance analysis and enrichments for flow cytometry or ATP measurement systems). It can also be used for the confirmation of suspicious single colonies from (selective) agar plates.

2.1 Media preparation and enrichment / BacTrac Screening

2.1.1 BacTrac screening (impedance analysis)

Note: For information regarding handling of specific SY-LAB media and regarding BacTrac protocols, please follow the respective SY-LAB media preparation instructions and BacTrac application notes for registered users on

our website (www.sylab.com, Microbiology, Service & Downloads / Electrical Microbiology and Molecular Microbiology sections).

The following pre-enrichment media are suitable for BacTrac analysis according to the respective SY-LAB BacTrac application notes:

- Casein-peptone lecithin polysorbate broth base (e.g. from Merck, product no. 1.11723.0500) with 40 ml / L polysorbate 80 (Tween® 80)
- TSB-TLHC Bouillon (e. g. Merck, product no. 1464920010)
- Eugon LT 100 Broth (e. g. Scharlau Microbiology , product no. 02-654)
- Modified Letheen Broth (e. g. Merck, product no. 1.10405.0500)

Protocol:

1. Prepare the required volume of pre-enrichment medium according to the instructions on the respective package and sterilize/autoclave.
TSB-TLHC bouillon is available ready-to-use in flasks of 90 ml.

Note: *Media containing polysorbate / lecithin should be removed from the autoclave while still warm (**Caution:** Handling of hot liquids involves danger of injury!) and be cooled **while stirring** on the magnetic stirrer, to ensure that the polysorbate / lecithin is homogenously dissolved (**Note:** Once solidified, the polysorbate cannot be dissolved anymore!).*

2. Homogenise 1 g of sample in 90 - 100 ml of pre-enrichment medium and incubate at +30 ± 1°C for 22 – 26 hrs.

3. Homogenise pre-enriched sample and transfer 1.0 ml into a pre-filled BacTrac measuring cell with 9.0 ml of BiMedia 001C.

4. Carry out BacTrac analysis using the specified measuring parameters.

Measuring parameters:	Temperature: +30°C
	Duration: 24 hrs.
	Delay time: 1 hour
	Interval time: 10 minutes
	Evaluation type: X
	Threshold M: 3%
	Threshold E: 10%

5. When the BacTrac analysis is finished, carry out standard protocol for RiboFlow® *Cosmetics* as described in section 2.2.1 (please observe the

important notes in sections 2.2 and 2.4). Only BacTrac-reactive, i.e. positive, samples are analysed.

Note: *This test should not be carried out right after the detection threshold has been surpassed in BacTrac impedance analysis, but only after a well-defined growth curve has developed.*

2.1.2 Direct enrichment, enrichment for flow cytometry and enrichment for ATP measurement systems

Note: For direct enrichment, the casein-peptone lecithin polysorbate broth described in 2.1.1 or enrichment media according to ISO 21249 or ISO 16212 can be used. For enrichments for flow cytometry or ATP measurement, the respective recommended special enrichment media are used.

Protocol:

1. Prepare the required volume of suitable enrichment broth according to the respective preparation instruction.

2. Homogenise 1 g of sample in 90 – 100 ml of enrichment broth and incubate at +30 ± 1°C for 22 – 48 hours, according to the method used.

3. Subsequently, carry out standard protocol for RiboFlow® *Cosmetics* as described in section 2.2.1 (please observe the important notes in sections 2.2 and 2.4). If flow cytometry or ATP measurement is applied, confirm only reactive (positive) samples using RiboFlow®.

2.1.3 Subcultivation of single colonies from agar plates

Notes: Single colonies with a diameter > 2 mm, or up to 5 pooled smaller single colonies of identical (suspicious) morphology can be taken directly from the agar plate and be tested in a RiboFlow® assay if desired (see 2.2.2, observe the important notes in sections 2.2 and 2.4), provided the plate was not incubated longer than the specified incubation time or stored.
If the plate is older and/or a colony to be tested is smaller, or if a single colony shall be tested in all four assays, sub-cultivation in liquid medium is necessary. Such a cultivation step will also allow for isolation of the bacterium by streaking it from the liquid culture to a new plate before the rest of the culture is

centrifuged for analysis. A colony taken from the plate and tested directly will not be available for isolation any more.

Protocol:

1. Streak bacteria on (selective) agar plates and incubate plates according to the method used.

2. Either analyse a big colony (> 2 mm) directly from the plate right after the specified incubation step, as described in protocol 2.2.2 (please observe the important notes above and in sections 2.2 and 2.4), using the respective RiboFlow® assay as desired, or perform sub-cultivation in a non-selective medium first, as described in step 3.

3. Sub-cultivation: With a sterile inoculation loop, pick 1 - 5 suspicious single colonies of identical morphology from the plate, transfer them into a sterile 2 ml reaction tube containing 2 ml of a non-selective medium (e. g. BHI medium), and incubate at +30 ± 1°C, until significant turbidity can be observed (8-24 hrs.).

4. Carry out RiboFlow® *Cosmetics* standard protocol according to 2.2.1 (please observe the important notes in sections 2.2 and 2.4).

2.2 RiboFlow® *Cosmetics* Lateral Flow Assays

Note: The measures for compliance with incubation / reaction conditions described in this protocol must be followed, otherwise results can be falsified. This includes the use of an IL-10 Mini-Incubator set to +48°C, the use of a pre-warmed RiboFlow® manipulation plate, as well as pre-warming of RiboFlow® lateral flow devices (test cassettes) and Solution C. Carry out manipulations swiftly and as far as possible inside of the Mini-Incubator with the door open, keep manipulations outside of the incubator (only with manipulation plate) to a minimum. Evaluation of the test result must be carried out immediately after lapse of the specified assay incubation time, before the lateral flow test cassettes can cool to ambient temperature.

2.2.1 Standard protocol for liquid cultures

Note: This protocol is suitable for enrichments and for other liquid cultures from non-selective media, e.g. sub-cultures of older / stored enrichments or of single colonies.

Protocol:

1. Set temperature of the IL-10 Mini-Incubator to +48°C and let incubator heat up until +48°C is stably displayed.

2. Pre-warm RiboFlow® manipulation plate in the incubator at +48°C for at least one hour before carrying out the assay.

Note: *The manipulation plate can also remain permanently in the (turned on) IL-10 Mini-Incubator instead of a shelf. In this case, you can start immediately with step 3. If the incubator was turned off, you have to wait one hour after +48°C is displayed stably before the assay is carried out, to ensure that the plate will also reach the required temperature.*

3. Pre-warm Solution C and the required RiboFlow® Lateral Flow Assay devices in the IL-10 Mini-Incubator set to +48°C for at least 10 minutes before the assay is carried out (one set of 4 devices each per sample = one device each for *E. coli, S. aureus, P. aeruginosa* and *C. albicans*). To this end, slide the devices into the pre-warmed RiboFlow® manipulation plate lying in the incubator, so that the sample ports are not covered by the acrylic glass cover of the plate (Figure 1).
Bring the other kit components / solutions to ambient temperature (+18 to +30°C) before use.

4. After cultivation, agitate liquid cultures gently (do not spill) or homogenise by pipetting up and down before taking samples for this test. For 2 ml sub-cultures of single colonies, start with the centrifugation step (step 6).

5. Transfer 2 ml of homogenised sample to a reaction tube.

6. Centrifuge bacteria for 5 minutes at a minimum of 2000 × g.

7. Carefully remove and discard supernatant without losing the bacterial pellet.

8. Resuspend bacterial pellet thoroughly but carefully in 200 µl of Solution A by pipetting up and down, avoid foaming.

9. Add 100 µl of Solution B to the sample and mix well (vortex if possible). Now the bacterial pellet must be completely resuspended.

Note: *If a vortex is available and several bacterial pellets have to be processed simultaneously, it may be more convenient and time-saving to add 300 µl each of a prepared 2 + 1 mixture of Solution A + Solution B to all samples first, and then to vortex the bacterial pellets thoroughly for resuspension.*

10. Incubate homogenisate at ambient temperature (+18 to +30°C) for 6 ± 1 minutes.

11. Add 240 µl of the pre-warmed Solution C to the sample and mix (vortex if possible). Proceed immediately with step 12.

12. Quickly apply 130 µl – aliquots each of a homogenised sample to the respective sample ports of the four pre-warmed RiboFlow® Lateral Flow Assay devices on the manipulation plate. Let the samples penetrate the sample application pads. Handle devices / manipulation plate carefully after application of samples to avoid spillage.

Notes: *Perform all necessary manipulations with the IL-10 Mini-Incubator nearby and avoid removing the RiboFlow® manipulation plate from the incubator before/during the assay. To avoid cooling of the plate and/or test cassettes before running the assay, the application of samples can be done conveniently and swiftly after sliding the plate slightly outwards on its mountings, with the incubator door open.*
Migration of the sample along the RiboFlow® device can conveniently be monitored through the glass window of the closed incubator door. If the flow does not start within 2 minutes (this can happen from time to time with extremely viscous samples), it may be helpful to scratch the surface of the pad in the sample port gently using a micropipette tip.

13. Incubate the assays on the manipulation plate for 15 ± 1 minutes in the closed IL-10 Mini-Incubator, then evaluate the result immediately (see section 2.3).

2.2.2 Standard protocol for direct testing of single colonies

Notes: This protocol is suitable for direct testing of single colonies (> 2 mm diameter) from plates, without additional sub-culturing in liquid medium. Instead of one big single colony, up to 5 typical (smaller) colonies of identical suspicious morphology can be pooled.
Please note that a sub-cultivation step as described in 2.1.3 must be carried out first if colonies are to be tested in more than one lateral flow assay and/or when plates were incubated too long or stored.

Protocol:

1. Set temperature of the IL-10 Mini-Incubator to +48°C and let incubator heat up until +48°C is stably displayed.

2. Pre-warm RiboFlow® manipulation plate in the incubator at +48°C for at least one hour before carrying out the assay.

Note: *The manipulation plate can also remain permanently in the (turned on) IL-10 Mini-Incubator instead of a shelf. In this case, you can start immediately with step 3. If the incubator was turned off, you have to wait one hour after +48°C is displayed stably before the assay is carried out, to ensure that the plate will also reach the required temperature.*

3. Pre-warm Solution C and the required RiboFlow® Lateral Flow Assay devices in the IL-10 Mini-Incubator set to +48°C for at least 10 minutes before the assay is carried out. To this end, slide the devices into the pre-warmed RiboFlow® manipulation plate lying in the incubator, so that sample ports are not covered by the acrylic glass cover of the plate (Figure 1).
Bring the other kit components / solutions to ambient temperature (+18 to +30°C) before use.

4. Prepare a mixture of 50 µl Solution A and 25 µl Solution B in an empty reaction tube.

5. Remove 1-5 typical colonies from the (selective) agar plate using an inoculation loop and resuspend thoroughly in the mixture of Solution A and Solution B prepared in paragraph 4.

6. Mix well (vortex if possible) and incubate mixture at ambient temperature (+18 to +30°C) for 6 ± 1 minutes.

7. Add 60 µl of the pre-warmed Solution C to the sample and mix. Proceed immediately with step 8.

8. Quickly apply the entire sample (~135 µl) to the sample port of the respective pre-warmed RiboFlow® Lateral Flow Assay device on the manipulation plate (*E. coli, S. aureus, P. aeruginosa*, or *C. albicans*, respectively, depending on what is tested). Let the sample penetrate the sample application pad. Handle device / manipulation plate carefully after application of sample to avoid spillage.

Notes: *Perform all necessary manipulations with the IL-10 Mini-Incubator nearby and avoid removing the RiboFlow® manipulation plate from the incubator before/during the assay. To avoid cooling of the plate and/or test cassettes before running the assay, the application of samples can be done conveniently and swiftly after sliding the plate slightly outwards on its mountings, with the incubator door open.*
Migration of the sample along the RiboFlow® device can conveniently be monitored through the glass window of the closed incubator door. If the flow does not start within 2 minutes (this can happen from time to time with extremely viscous samples), it may be helpful to scratch the surface of the pad in the sample port gently using a micropipette tip.

9. Incubate assay(s) on the manipulation plate for 15 ± 1 minutes in the closed IL-10 Mini-Incubator, then evaluate the result immediately (see section 2.3).

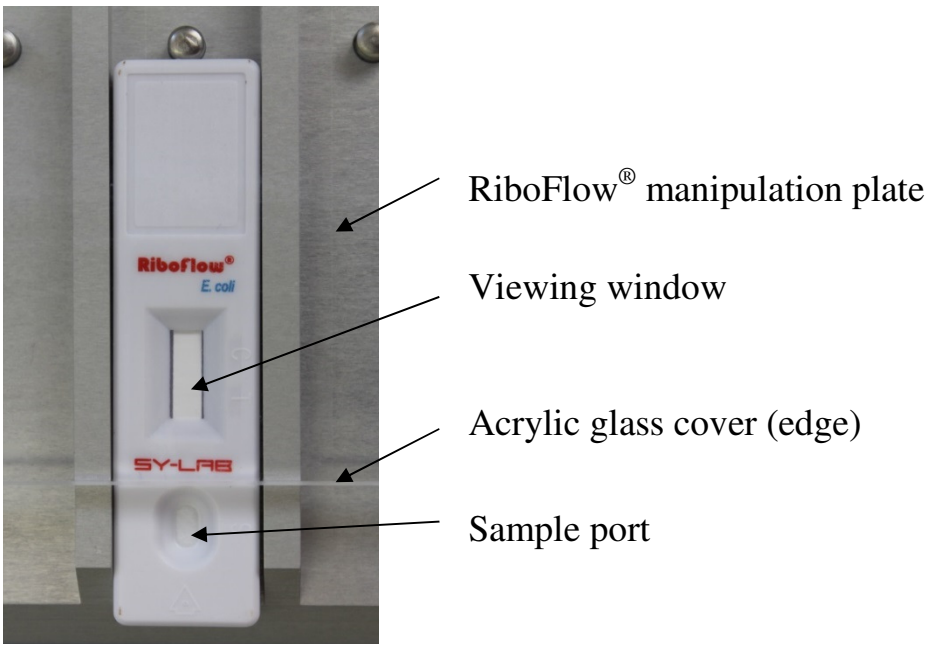

Fig. 1: RiboFlow® Lateral Flow Assay device (example: *E. coli*)

2.3 Evaluation

Note: RiboFlow® lateral flow assays must be evaluated **immediately** after 15 ± 1 minutes runtime. Runtimes >16 minutes might lead to false positive results, especially when the temperature during the run was too low. Unspecific lines may also appear a few minutes after the test is finished and is cooling to ambient temperature.

Evaluation: Figs. 2 - 4 are showing viewing windows displaying possible results of RiboFlow® lateral flow assays.

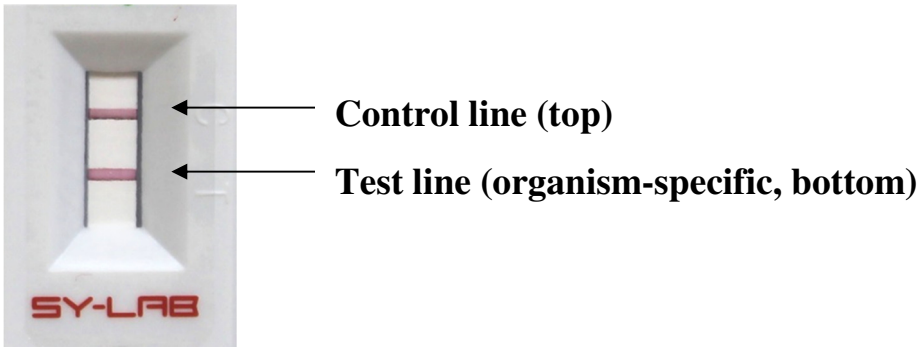

Fig. 2: Positive results:
If both control line (top) and the organism-specific test line (bottom) are visible in the viewing window of the respective assay, the result is positive for this organism.

Sometimes the control line (top) is very faint in strongly positive samples.

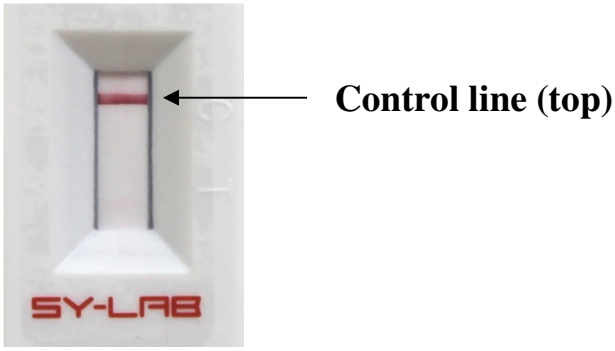

Fig. 3: Negative results:
If only the control line (top) is visible, no target organism could be detected with the assay, and the result is negative.

Fig. 4: Invalid result: If no lines are visible at all, some error has occurred during processing of the assay. Such a result is invalid, and the test has to be repeated starting from section 2.2, using a new RiboFlow® lateral flow assay device.

To file a permanent record of results (if desired), we recommend photography using a digital camera immediately at the end of the runtime. Since the acrylic glass cover of the RiboFlow® manipulation plate is transparent, the devices can be conveniently left in the plate for photography. This will also delay cooling of the devices and thus the potential danger of formation of false positive signals after the run.

2.4 Important general notes

- This assay should always be carried out with freshly grown bacteria at the end of the specified incubation time for the respective enrichment/culture. It should not be used with stored/old samples or enrichments/plates that were incubated for too long, since rRNA may be degraded during prolonged incubation or storage, potentially declining to undetectable levels. However, the rest of an enriched sample (BacTrac measuring cell or enrichment) can be stored refrigerated for a few hours until a result is available, to enable a second analysis on the same day, if necessary. If longer storage of an enriched sample is unavoidable, a sub-culture using a non-selective medium should be performed to boost rRNA synthesis prior to analysis.
  Bacterial pellets after centrifugation or bacteria resuspended in Solution A may be stored as pellets or lysates at -20°C for prolonged time (a few weeks).

- Always agitate enriched cultures gently without spilling, or homogenise by pipetting up and down before taking a sample.

- The bacterial pellet must be completely and homogenously resuspended after mixing with Solution B!

- Centrifugations must be carried out for at least 5 minutes with a relative centrifugal force (RCF) of at least 2000 × g (up to 5000 × g) to ensure sedimentation of bacteria. Information regarding the RCF can be found in the user manual of your microcentrifuge.

- When several samples are analysed simultaneously, it is advisable to keep time in-between working steps as short as possible (not more than 1 minute), especially after the incubation step with Solution B.

- The specificity of a nucleic acid hybridisation assay is strongly dependent on temperature, especially when no washing step is performed, as in a lateral flow assay setting, and when closely related species have to be discriminated. The RiboFlow® *Cosmetics* Detection Kit was developed and evaluated/validated under the conditions stated in this manual and using an IL-10 Mini-Incubator and a RiboFlow® manipulation plate. If other incubators, which have not been specifically qualified, are used, false positive results may occur.

- Lateral flow assays must be evaluated **immediately** and **quickly** after lapse of the specified runtime, since cooling to ambient temperature may falsify negative results.

- Always work with sterile pipette tips to avoid microbial or nuclease contamination of kit components.

- RiboFlow® **video tutorials** are available on our website (www.sylab.com, Microbiology, Service & Downloads / Molecular Microbiology section) for proper guidance! If you have further questions concerning this kit the SY-LAB customer support will be glad to assist you.

3. Ordering information

- **RiboFlow® *Cosmetics* Detection Kit**, 6 × 4 assays, product number 51-420113
- **BiMedia 001C,** 120 pre-filled measuring cells, product number 41-440011
- **Mini-Centrifuge M08**, product number 51-410000
- **Mini-Incubator IL10**, product number 51-410100
- **RiboFlow® manipulation plate**, product number 51-410110

4. Quick reference protocols

4.1 Quick reference protocol for liquid cultures

Step		Duration
1.	Enrichment[1]	22 - 48 hrs
2.	Pre-warm lateral flow assay devices (on the pre-warmed RiboFlow® manipulation plate) and Solution C for at least 10 min in an IL-10 Mini-Incubator set to +48°C. Bring other kit components to ambient temperature (+18 to +30°C).	
3.	Centrifuge 2 ml of enriched sample from measuring cell[2] or from enrichment[3]	~5 min
4.	Remove supernatant and resuspend bacteria in 200 µl of Solution A	~1 min
5.	Add 100 µl of Solution B, mix and incubate at ambient temperature (+18 to +30°C)	6 ± 1 min
6.	Add 240 µl of pre-warmed Solution C and mix	~0.5 min
7.	Apply 130 µl - aliquots of the sample to the 4 pre-warmed RiboFlow® lateral flow assays on the manipulation plate in the IL-10 Mini-Incubator set to +48°C and incubate	15 ± 1 min
8.	Evaluate result immediately	

[1]Pre-enrichment + BacTrac analysis, direct enrichment, enrichment for flow cytometry, or enrichment for ATP measurement
[2]Only BacTrac-reactive (positive) samples are analysed
[3]In case of flow cytometry or ATP measurement, only reactive (positive) samples are analysed, in case of direct enrichment all samples are analysed.

4.2 Quick reference protocol for direct testing of single colonies

Step		Duration
1.	Streak and incubate plate acc. to method used	24 - 48 Std.
2.	Pre-warm lateral flow assay devices (on the pre-warmed RiboFlow® manipulation plate) and Solution C for at least 10 min in an IL-10 Mini-Incubator set to +48°C. Bring other kit components to ambient temperature (+18 to +30°C).	
3.	Prepare a mixture of 50 µl Solution A and 25 µl of Solution B in a reaction tube	~1 min
4.	Thoroughly resuspend a single colony (> 2 mm diameter, or up to 5 morphologically identical suspicious colonies) in the prepared mixture	~0,5 min
5.	Incubate at ambient temperature (+18 to +30°C) for 6 ± 1 minutes	6 ± 1 min
6.	Add 60 µl of pre-warmed Solution C and mix	~0.5 min
7.	Apply entire sample (apprx. 135 µl) to pre-warmed RiboFlow® lateral flow assay device on the manipulation plate in the IL-10 Mini-Incubator set to +48°C and incubate	15 ± 1 min
8.	Evaluate result immediately	