

Phusion[®]



Bacterial Profiling Kit

F-890

Phusion® Bacterial Profiling Kit

Instruction manual

F-890S Sufficient for 100 reactions

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1. Introduction

Finzymes' Phusion® Bacterial Profiling Kit has been developed for the profiling and characterization of bacterial populations by means of terminal restriction fragment length polymorphism (T-RFLP). T-RFLP allows the fingerprinting of a bacterial community by analyzing the polymorphism of a certain gene.

The protocol of the Phusion Bacterial Profiling Kit is outlined in Figure 1. First, polymorphic segments of the bacterial 16S ribosomal RNA gene are amplified by PCR using fluorescently labeled primers. The PCR reaction is designed to produce fragments of 900–950 nucleotides, which are subsequently cleaved by adding a restriction endonuclease solution directly to the PCR product. The cleaved products are then separated and detected using a capillary electrophoresis instrument. The Phusion Bacterial Profiling Kit produces distinct profiles (fingerprints) dependent on the species composition of the bacterial communities in the sample. The bacterial strains and species are characterized using a tailor-made internet database of T-RFLP peak values. A user name and password for access to this database are included with this kit. The Phusion Bacterial Profiling Kit also includes a Microbe Control DNA mixture for verifying successful reaction conditions in the PCR, endonuclease digestion and electrophoresis steps.

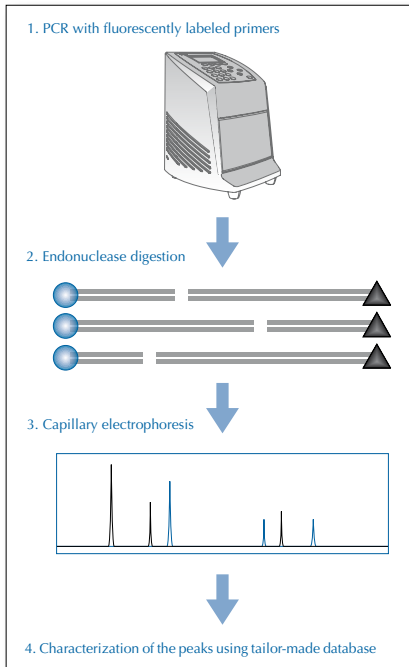


Figure 1. Workflow of the Phusion® Bacterial Profiling Kit protocol.

The Phusion Bacterial Profiling Kit offers several technical advantages over other T-RFLP-based protocols. The highly processive proofreading Phusion® Hot Start High-Fidelity DNA Polymerase provides robust and consistent amplification. Furthermore, this polymerase is more tolerant of PCR inhibitors present in many samples.

In the Phusion Bacterial Profiling Kit protocol, the restriction enzyme solution is directly added to the PCR product, which minimizes the cross-contamination risk and circumvents purification steps. The endonuclease solution of the Phusion Bacterial Profiling Kit contains agents to block residual polymerase activity (fill-in reactions of 5'-overhangs and plus-A activity). These blocking agents minimize the risk of split peaks of 1–3 nt size difference to yield single, correctly sized peaks in the electrophoretic profile.

2. Kit components

Component	Description
Microbe PCR solution 1 contains Phusion® Hot Start High-Fidelity DNA Polymerase, dNTPs and Phusion Bacterial PCR buffer with MgCl ₂	100 reactions; blue cap
Microbe PCR Solution 2 contains the following differentially labeled fluorescent PCR primers: 5' AGA GTT TGA TCC TGG CTC AG 3' (blue)* 5' CCG TCA ATT CCT TTR AGT TT 3' (yellow)*	100 reactions; red cap
Endonuclease Solution MT1 contains <i>MspI</i> and <i>HinPII</i> endonucleases**	100 reactions; colorless cap
Microbe Control DNA contains a mixture of DNA from the following 5 bacterial strains: <i>Lactococcus</i> sp., <i>Staphylococcus aureus</i> , <i>Streptococcus agalactiae</i> , <i>Enterococcus faecalis</i> and <i>Pasteurella dagmatis</i>	10 reactions; green cap
User name and password for the Phusion® Bacterial Profiling Kit Database	Card

* Dye colors as they appear after electrophoresis with Dye Set G5 (Applied Biosystems)

** Recognition sequences: *MspI*, C'CGG; *HinPII*, G'CGC

Material safety data sheet (MSDS) is available at www.finnzymes.com.

3. Shipping and storage

The Phusion Bacterial Profiling Kit is shipped on gel ice. Upon arrival, store all kit components at -20°C. Reagents can be refrozen after use. Always store Microbe PCR Solution 2 protected from light. The kit is stable for six months from the packaging date if stored and handled properly.

4. PCR

Use purified DNA for the PCR. DNA extraction can be performed by using any suitable protocol. After thawing, keep the Microbe PCR Solutions 1 and 2 on ice during reaction setup. Refreeze the reagent stocks immediately once the PCR reaction has been set up. The reagents tolerate several freeze-thaw cycles.

1. Prepare a PCR premix by combining equal amounts of **Microbe PCR Solution 1** and **Microbe PCR solution 2**.

- Volume of **Microbe PCR Solution 1** = $N \times 9.5 \mu\text{l}$
- Volume of **Microbe PCR Solution 2** = $N \times 9.5 \mu\text{l}$

N = Number of samples

Including the following controls:

- positive control (Microbe Control DNA)
- negative control (H_2O)

The mix provides excess volume to compensate for loss in pipetting.

2. Mix by inverting five times or by vortexing for 5 seconds. Spin down briefly.
3. Into each PCR reaction tube, add
 - 18 μl of the PCR premix
 - 2 μl of either sample DNA extract (max. 100 ng), Microbe Control DNA (positive control), or H_2O (negative control).
4. Mix briefly, spin down and place the reaction vessels in a thermal cycler. Run the PCR program provided in Table 1. After the PCR, keep the vessels refrigerated or proceed directly to DNA digestion.

Table 1. Cycling instructions.

Cycle step	Temperature	Time	Number of cycles
Initial denaturation	98°C	3 min	1
Denaturation	98°C	20 s	30**
Annealing	59°C	30 s*	
Extension	72°C	30 s	
Final extension	72°C 4°C	5 min hold	1

* Can be extended up to 60 s for samples of poor quality (those containing high amounts of PCR inhibitors, for example)

** Can be increased up to 35 cycles for samples of poor quality or samples with low amount of DNA

Use the full ramping speed and calculated temperature control functions of the thermal cycler, if adjustable.

5. Endonuclease digestion

1. Add 2.5 µl of Endonuclease Solution MT1 into each PCR reaction.

Note: Endonuclease Solution MT1 is provided in 50 % glycerol and thus remains in liquid form at -20°C. The solution can be used immediately without thawing. The contents of the tube should be mixed well by inverting the tube several times. Endonuclease solution MT1 should be pipetted slowly and the pipette tip submerged as little as possible below the surface, because the viscosity of glycerol may otherwise lead to pipetting errors.

2. Mix by pipetting in and out.
3. Incubate for 2 hours at 37°C.

The samples can be stored at +4°C up to a few hours or at -20°C for longer periods.

6. Capillary electrophoresis

6.1 General information

The fragment analysis of the Phusion Bacterial Profiling Kit works optimally with ABI PRISM® Genetic Analyzers (e.g. models 310, 3100, 3130 and 3730; Applied Biosystems). In addition to the instructions below, please refer to the instrument user guide.

The Phusion Bacterial Profiling Kit is compatible with Dye Set G5 (Applied Biosystems), which requires matrix files generated with the Matrix Standard Set DS-33 (Dye Set G5). The matrix file values vary between instruments and electrophoresis conditions. A separate matrix file must therefore be generated for each instrument.

Because the fragments obtained with the Phusion Bacterial Profiling Kit can be up to 900 nucleotides long, we strongly recommend using the GeneScan™ LIZ® 1200 Size Standard (Applied Biosystems). Run modules for the GeneScan 1200 LIZ Size Standard can be downloaded from www.appliedbiosystems.com. Note that the run times may need to be extended from those present in the run modules in order to score all fragments.

The quantity of the PCR products varies depending on the amount and quality of the DNA template. We recommend preparing a dilution series of the PCR products and running electrophoresis in order to optimize the peak intensities in the electrophoresis.

6.2 Data analysis

The electrophoretic profile of the sample is determined using GeneScan*, GeneMapper®, Peak Scanner™ (all Applied Biosystems) or any other software designed to calculate fragment sizes by comparison to a size standard.

In order to obtain a more accurate sizing of the sample fragments, it is necessary to edit the size standard definition of the GeneScan 1200 LIZ Size Standard in the analysis software. Editing is needed because a slight sizing error occurs in the capillary electrophoresis due to the difference in the DNA composition between the 16S rRNA gene and the size standard.

Edit the size standard definition in the software by changing the peak size values of the GeneScan LIZ1200 Size Standard fragments according to the instruction manual of your fragment analysis software. Use the values shown in Table 2. The software assigns the new size standard values to the peaks of the size standard and calculates the fragment sizes in the sample using this information. The new size standard definition values provided in Table 2 are optimized for the Phusion Bacterial Profiling Kit.

* **Note:** In order to handle the size range of the GeneScan LIZ 1200 Size Standard, it is necessary to use GeneScan version 3.7.1. It contains the Long Fragment Enabler, a batch file which allows the user to toggle back and forth between standard and large fragment applications. GeneScan v3.7 cannot handle the size range of the GeneScan LIZ 1200 Size Standard. Download the GeneScan v3.7.1 update from www.appliedbiosystems.com.

Table 2. Default and edited GeneScan™ LIZ® 1200 Size Standard fragment size values. In your fragment analysis software, replace the values in the first column with the “Edited values” in the second column. This table can be downloaded from www.finnzymes.com/phusionbact.

A. Values for the blue peaks*

Default value	Edited value	Default value	Edited value	Default value	Edited value	Default value	Edited value
20	33	240	246	440	447	660	667
40	50	250	255	460	467	680	687
60	68	260	265	480	487	700	707
80	88	280	286	500	507	714	721
100	108	300	306	514	521	720	727
114	121	314	320	520	526	740	747
120	126	320	326	540	545	760	767
140	146	340	346	560	566	780	787
160	166	360	367	580	587	800	807
180	186	380	387	600	607	814	821
200	205	400	408	614	621	820	827
214	221	414	422	620	627	840	847
220	225	420	427	640	647	850	857

B. Values for the yellow peaks*

Default value	Edited value	Default value	Edited value	Default value	Edited value	Default value	Edited value
20	34	240	245	440	447	660	662
40	49	250	254	460	467	680	682
60	67	260	265	480	487	700	702
80	87	280	286	500	507	714	716
100	106	300	306	514	521	720	723
114	120	314	320	520	526	740	744
120	126	320	326	540	545	760	763
140	145	340	346	560	562	780	782
160	164	360	367	580	582	800	802
180	185	380	386	600	602	814	816
200	205	400	407	614	616	820	822
214	220	414	421	620	622	840	842
220	225	420	426	640	642	850	852

* Dye colors as they appear after electrophoresis with Dye Set G5 (Applied Biosystems).

6.3 The electrophoretic profile of the Microbe Control DNA

Figure 2 shows the fragment sizes of the Microbe Control DNA. Panel B demonstrates that using the values optimized for the Phusion Bacterial Profiling Kit (Table 2) results in accurate sizing of the fragments.

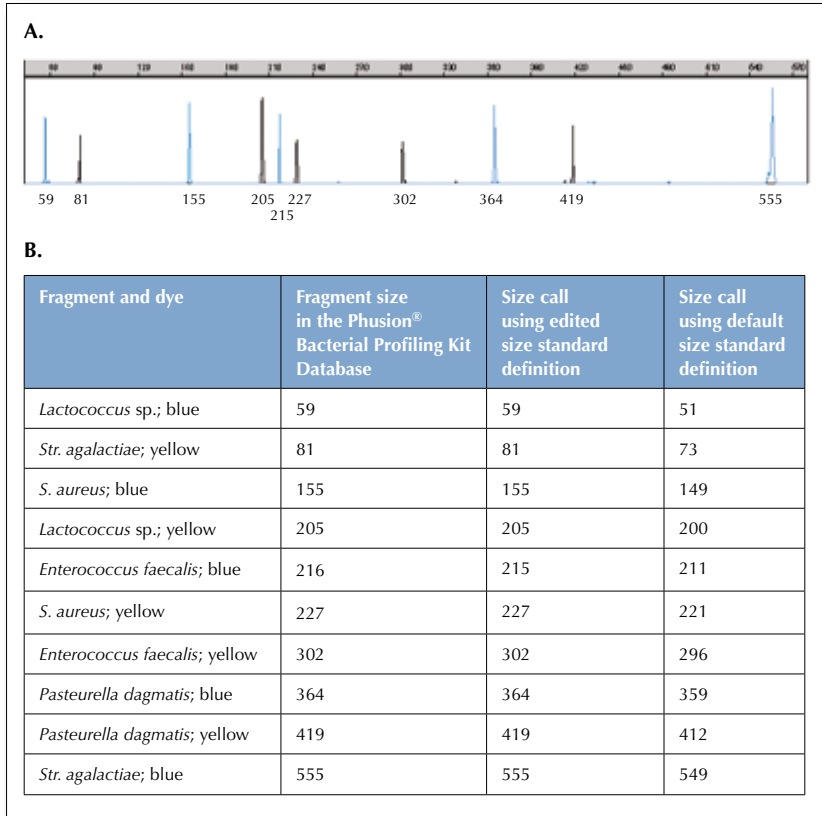


Figure 2. Microbe Control DNA. **(A)** Electrophoretic profile of the amplified and digested Microbe Control DNA obtained with the Phusion® Bacterial Profiling Kit using the edited size standard definition. **(B)** Microbe Control DNA fragment length values in the Phusion® Bacterial Profiling Kit Database (first column) and the values obtained using edited (second column) and default (third column) fragment size definition of the GeneScan™ 1200 LIZ® Size Standard.

7. Characterization of the bacterial species and strains

Bacterial species and strains are characterized using the Phusion Bacterial Profiling Kit Database (Figure 3). This regularly updated database is tailor-made for this kit. It is based on 16S ribosomal RNA gene sequences of more than 60 000 targets.

Access to this database is included with the kit. In the kit box, you will find a card with a user name and a password for the database. The user name is valid for the indicated stability period of the kit. To log in to the database, open the URL www.finnzymes.com/phusionbact in your browser and type in the user name and password.

In the form that appears, type the values of the blue peak and the corresponding yellow peak in the fields.* Make sure to use the size calls obtained using the edited size standard definition as instructed in Section 6.2. Include some margin in your search. For example, for a size call of 125.07, search for values between 124 and 126. For larger restriction fragments, the size calls may differ by up to several nucleotides from the values obtained from the capillary electrophoresis instrument software.

The database lists the bacterial species or subgroups matching your search. You can sort the results in ascending or descending order by clicking any of the column titles. Click the locus name in the Hyperlink column to access the NCBI GenBank entry for the given microorganism. To print the list, click “Printable version”.



Phusion[®] Bacterial Profiling Kit Database

Blue* peak value (nt)

Blue peak value (nt) (nt)

Yellow* peak value (nt)

Yellow peak value (nt)

I want to sort my results by:

Do not see what you want? Sort the results in ascending or descending order.

Accession	Organism	Length Blue	Length Yellow	Hyperlink
U00096.1	Escherichia coli (E. coli)	144	144	U00096.1
AF151480	Escherichia coli (E. coli) strain ATCC 8739	144	144	AF151480
AF021129	Escherichia coli (E. coli) strain ATCC 8739	144	144	AF021129
U00096.1	Escherichia coli (E. coli)	144	144	U00096.1
U00096.1	Escherichia coli (E. coli)	144	144	U00096.1
AF151480	Escherichia coli (E. coli) strain ATCC 8739	144	144	AF151480

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Figure 3. Access to the tailor-made database is included with the kit. Type the value of the blue peak and the corresponding yellow peak in the fields. The database lists the bacterial species or subgroups matching your search.

* Dye colors as they appear after electrophoresis with Dye Set G5 (Applied Biosystems).

8. Troubleshooting

Problem	Possible explanation	Recommended action
Faint or no signals from the test sample but normal signals from the Microbe Control DNA.	Concentration of PCR inhibitors in the sample too high	Dilute the PCR template in distilled water and repeat the protocol.
	Low amount of template DNA	Add 5 more cycles to the PCR program and repeat the protocol.
	Template DNA amount too high	Titrate the template amount. Do not exceed 100 ng of template DNA.
Faint or no signals from both the test sample and the Microbe Control DNA.	Error in the PCR setup or protocol	Check the PCR program and repeat the protocol.
Additional signals (peaks) appearing at the same sizes in both the test samples and the Microbe Control DNA.	PCR carryover contamination	Follow the instructions in Appendix 1 to avoid contamination and repeat the protocol.
	Excessive DNA digestion time	Make sure not to exceed 3 h of DNA digestion (see Chapter 5).
	Digested product stored too long before electrophoresis	Perform electrophoresis within hours after DNA digestion or freeze the digested DNA until electrophoresis.
Split peaks in the Microbe Control DNA and test samples.	Temperature too high during DNA digestion	Make sure that the incubation temperature is 37°C ($\pm 1^\circ\text{C}$).
The fragment size values obtained from electrophoresis differ from the expected values.	The size standard definition was not edited	Make sure to redefine the size standard before comparing the sample fragments with the database (see Section 6.2).
Additional peaks in the electrophoretic profile of the Microbe Control DNA.	PCR carryover contamination	Follow the instructions in Appendix 1 to avoid contamination and repeat the protocol.
	Incomplete DNA digestion	Add 0.5–1 hour to the incubation time (see Chapter 5). However, do not exceed 3 h.

Appendix I: Avoiding PCR carryover contamination

Due to their extreme sensitivity, PCR assays are susceptible to carryover contamination by previously amplified PCR products. A single molecule of amplified DNA may influence the results by contaminating the reaction mixture before PCR.

In order to minimize such risks, follow these guidelines:

- Always wear fresh gloves.
- Use aerosol-resistant pipette tips.
- Use new and/or sterilized glassware and plasticware.
- Designate physically separated working areas for: (1A) DNA extraction and endonuclease digestion, (1B) setting up the PCR reactions, and (2) preparing electrophoresis reagent mixes and performing capillary electrophoresis. If possible, designate three separate areas for (1A), (1B) and (2), but in any case make sure to separate (1) and (2). Workflow in the laboratory should always proceed unidirectionally from (1) to (2) and traffic from the electrophoresis area to the other separated areas during the same day should be avoided.
- Use different laboratory equipment (disposable gloves, micropipettes, pipette tip boxes, laboratory coats etc.) in each working area.
- Change gloves frequently and always before leaving an area.

Warranty

Finnzymes Oy warrants that its products will meet the specifications stated on the technical data section of the data sheets, and Finnzymes Oy agrees to replace the products free of charge if the products do not conform to the specifications. Notice for replacement must be given within 60 days of receipt. In consideration of the above commitments by Finnzymes Oy, the buyer agrees to and accepts the following conditions:

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3. That the buyer's sole remedy shall be to obtain replacement of the product free of charge from Finnzymes Oy; and
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Trademark and license information

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Phusion® Bacterial Profiling Kit has been developed in collaboration with Dr. Van Haeringen Laboratorium, BV. This product is sold under license from Affibody AB, Sweden.

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