Product Manual

AMPIPROBE® Bacterial Vaginosis Assay Kit

Catalog #: ENZ-GEN205-0100

100 Assays

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Product Manual

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Please read entire booklet before proceeding with the assay.

Carefully note the handling and storage conditions of each kit component.

Please contact Enzo Life Sciences Technical Support if necessary.

DESCRIPTION

The AMPIPROBE® Bacterial Vaginosis Assay is a real-time polymerase chain reaction (qPCR) assay for the semi-quantitative detection of *Atopobium vaginae, Gardnerella vaginalis, Lactobacillus spp.*, *Megasphaera spp., and* BVAB2 genomic DNA. The kit uses the AMPIPROBE® assay platform which takes advantage of paired fluorophore- and quencher-labeled primers specific for each of the target species as well as an internal control. The kit contains all reagents necessary for PCR-based detection of *Atopobium vaginae, Gardnerella vaginalis, Lactobacillus spp., Megasphaera spp., and* BVAB2 DNA. In addition, the assay kit includes 3 positive quantitative PCR controls consisting of a mixture of the target templates at varying concentrations and a no template control. Please read the complete kit insert before performing this assay.

INTRODUCTION

Bacterial vaginosis (BV) is the most common vaginal infection in the United States and affects approximately 29% of women.[1] BV complications include an increased risk to develop other sexually transmitted infections and an increased risk of infection after pelvic surgery.[2] In addition, BV during pregnancy has been linked with adverse pregnancy outcomes including preterm labor and delivery, low birth weight, premature rupture of membranes, postpartum metritis and intra-amniotic infection. [3, 4]

Clinical signs and symptoms (Amsel criteria) are often used to diagnose BV but the gold standard method for diagnosis is enumeration of bacterial morphotypes on a Gram-stained smear.[5,6]

Although the exact etiology of BV is still undetermined, the consensus is that it occurs when *Lactobacillus spp*., which normally comprising 90-95% of total bacteria in healthy vaginal flora, is replaced by anaerobes, mainly but not restricted to *Gardnerella vaginalis*.[7,8] More recently, molecular detection of other microbes, that are mostly uncultureable, have been associated with BV including *Atopobium vaginae*, BVAB2, and *Megasphaera spp*.[9,10]

AMPIPROBE® TECHNOLOGY

Enzo's AMPIPROBE® technology incorporates probe detection technology in primer design. It employs a combination of fluorescent reporter-labeled primers and quencher-labeled primers to amplify DNA, akin to traditional PCR. When free in solution, fluorescent primers generate a signal. However, as the primers are incorporated into amplified DNA, the quencher and the fluorophore are brought within close proximity and exhibit Förster resonance energy transfer (FRET). This causes a logarithmic decay of signal with respect to the number of PCR amplification cycles. The cycle number at which fluorescence signal drops below a defined threshold, is indicative of the amount of target nucleic acid present in the sample.

Enzo's AMPIPROBE® Assay kits provide the following benefits:

- High sensitivity
- Low sample input
- Adaptable to multiplexing
- Compatible with open qPCR instruments

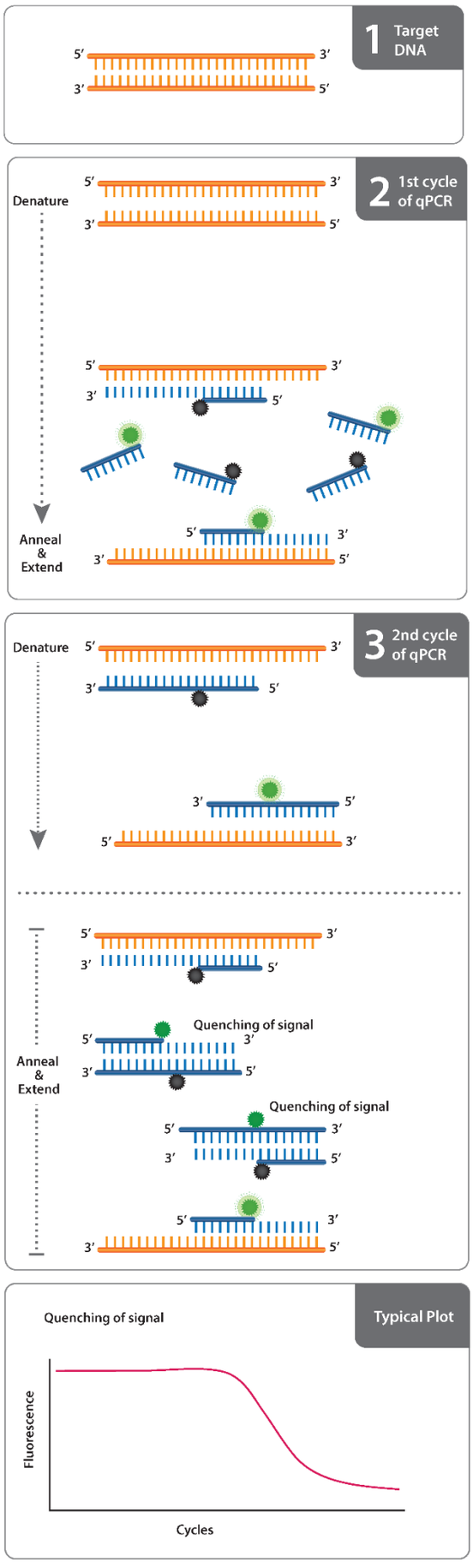
PRINCIPLE OF AMPIPROBE® BACTERIAL VAGINOSIS ASSAY

The AMPIPROBE® Bacterial Vaginosis (BV) Assay is a real-time PCR assay for the detection of *Atopobium vaginae, Gardnerella vaginalis, Lactobacillus spp., Megasphaera spp., and* BVAB2*.* The nucleotide sequences of the PCR primers have been optimized to target sequences within species-specific regions of the target genome. The BV primers have been designed using AMPIPROBE® technology which incorporates reporter and quencher dyes into the primers. Target detection is based on fluorescence decay when successive rounds of amplification bring fluorophore and quencher-labeled primer pairs in close proximity resulting in FRET. Loss of fluorescence below a defined threshold in a particular channel indicates sample positivity for the corresponding BV species.

The AMPIPROBE® BV Primer Mixes include primers for a ubiquitously conserved human housekeeping gene (human ß-globin). Successful amplification of the internal control serves as an indicator of sample adequacy, extraction efficiency and successful amplification in each individual sample.

The AMPIPROBE® Bacterial Vaginosis Assay is a two tube assay and contains two separate BV primer mixes. Both BV primer mixes must be run with each sample in order to determine the positive or negative status of the sample.

AMPIPROBE® TECHNOLOGY SCHEMATIC



SAFETY WARNINGS & PRECAUTIONS

Avoid freeze / thaw cycles

Handle with care

FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

- Kit components should be stored at or below -20°C. Care should be taken to limit the number of freeze-thaw cycles.
- The AMPIPROBE® BV Primer Mixes contain fluorescently labeled primers. To avoid photobelaching, protect from prolonged exposure to light.
- Some components of this kit may contain hazardous substances. Reagents can be harmful if ingested or absorbed through the skin and may cause irritation to the eyes. Reagents should be treated as possible mutagens and should be handled with care and disposed of properly.
- Practice aseptic technique when handling reagents to avoid introduction of contaminants that might interefere with assay interpretation.
- The use of screw-cap tubes and barrier pipette tips is strongly encouraged to prevent samples and reagents from becoming aerosolized which might lead to contamination.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas. Any biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

MATERIALS SUPPLIED

Reagent	Quantity
AMPIGENE® HS Taq DNA Polymerase	2 x 100 µL
AMPIGENE® dNTP Mix	2 x 50 µL
AMPIPROBE® 5X Assay Buffer	2 x 500 µL
AMPIPROBE® BV I Primer Mix	500 µL
AMPIPROBE® BV II Primer Mix	500 µL
BV Control 1	200 µL
BV Control 2	200 µL
BV Control 3	200 µL
No Template Control (NTC)	200 µL
Nuclease-free Water	2 x 1 mL

STORAGE

All components of this kit are stable at -20°C until the kit's expiration date.

OTHER MATERIALS REQUIRED BUT NOT SUPPLIED

1. Vortex mixer
2. Mini centrifuge
3. Calibrated pipettes capable of delivering volumes between 1 and 1000 µL
4. DNase/RNase free barrier pipette tips
5. DNase/RNase free 1.5 mL Screw Cap Micro tube (for preparation of master mix)
6. qPCR instrument and compatible accessories

PROCEDURAL NOTES

Do not mix components from different kit lots or use reagents beyond the kit expiration date.

SAMPLE COMPATIBILITY

Sample types and DNA extraction methods should be evaluated for compatibility with the AMPIPROBE® Bacterial Vaginosis Assay Kit as part of the user's validation process.

REAGENT PREPARATION

1. Pre-cool Loading Block at 4°C for at least one hour prior to setup.
2. Thaw the following kit reagents at room temperature (15-25ºC): Nuclease-free Water, AMPIGENE® dNTP Mix, AMPIPROBE® 5X Assay Buffer, AMPIPROBE® BV I Primer Mix, AMPIPROBE® BV II Primer Mix, BV Control 1, BV Control 2, BV Control 3, and NTC Control.

   Once thawed, vortex the reagents to mix. Spin briefly to collect the contents at the bottom of tubes. Keep the reagents on ice until ready to use. Protect the AMPIPROBE® BV Primer Mixes from prolonged exposure to light.

   Note: Some precipitation may occur in the AMPIPROBE® 5X Assay Buffer. Vortex and warm (37°C) vial to dissolve precipitate prior to use.
3. Remove the AMPIGENE® HS Taq DNA Polymerase from storage and briefly spin to collect contents at the bottom of tube. **Do not vortex**. Keep tube on ice until ready to use.

PCR SETUP

The procedure described below uses the Qiagen Rotor-Gene Q instrument, but other real-time PCR instruments capable of detecting fluorescence quenching may also be used. Refer to the manufacturer's instrument manual for details regarding operation of the PCR machine.

For each assay run, include the following controls: (a) BV Control 1, (b) BV Control 2, (c) BV Control 3, (d) NTC. Depending upon the user's requirements, additional controls may be needed. Each of the controls and the samples must be run with both BV Primer Mixes in two separate tubes.

Note: Due to differences in primer fluorescence, BV I and BV II Primer Mixes should not be in the same run or the gain should be set on each individually.

1. Immediately prior to use, prepare sufficient **PCR Master Mix** for the DNA samples and controls in a labeled, DNase/RNase free screw-cap tube, according to the volumes given in **Table 1** and **Table 2**. Add the reagents to the tube in the order listed. Include sufficient volume for 2 extra reactions to allow for any pipetting variance.

The **PCR Master Mix** contains all of the components needed for the reaction except the DNA template (sample).

2. Thoroughly mix the **PCR Master Mix** by either inverting the tube or by pipetting up and down until swirls are no longer visible (10-20 times). **Do not vortex**. Centrifuge briefly to bring contents to the bottom of the tube. Avoid prolonged exposure to light.
3. Dispense 15 µL of **PCR Master Mix** into 0.1 mL PCR tubes, taking care to deliver the solution to the bottom of the tube.
4. Add 10 µL sample or control to the PCR tube and mix by pipetting up and down at least 3 times.
5. Cap the tubes and visually inspect to confirm a tight seal and correct volume.
6. Place the tubes in the thermal cycler and run the method. See **PCR RUN PROFILE** section for the method details.

Table 1. Preparation of PCR Master Mix

Reagent	Volume per reaction	Total volume required [a,b]
Nuclease-free Water	3.5 µL	3.5 µL x (N+2)
AMPIPROBE® 5X Assay Buffer	5 µL	5 µL x (N+2)
AMPIPROBE® dNTP Mix	0.5 µL	0.5 µL x (N+2)
AMPIPROBE® BV I-II Primer Mix	5.0 µL	5.0 µL x (N+2)
AMPIGENE® HS Taq DNA Polymerase	1 µL	1 µL x (N+2)
TOTAL	**15 µL**	**15 µL x (N+2)**

Note: Use this table to prepare the Master Mix for Tube 1 and Tube 2. In Tube 1 include AMPIPROBE® BV I Primer Mix and in Tube 2 include AMPIPROBE® BV II Primer Mix.

[a] *Total volume required for **N + 2** reactions, where **N** is the number of samples plus controls. When preparing the Master Mix, prepare enough for 2 extra reactions to allow for any pipetting variance.*

[b] *If the number of samples plus controls exceeds 24, more than 2 extra reaction volumes may be needed.*

PCR RUN PROFILE

Create a temperature profile on your PCR instrument as indicated in **Table 2** and **Table 3**. The method described has been verified using a Qiagen Rotor-Gene Q instrument.

Table 2. PCR Cycling Parameters for Tube 1

Step	Parameter	Temp [°C]	Cycles	Hold [mm:ss]
Hold	Enzyme Activation	95°	1	3:00
Cycling	Amplification	95°	40	00:10
		68°		00:30 (Acquiring to Cycling A on Green, Yellow, Orange, Red)

Fluorescence is detected in the **FAM**/Green, **HEX**/Yellow, **ROX**/Orange and **Cy5**/Red channels at the end of the 2nd segment of the cycling step (68°C).

Table 3. PCR Cycling Parameters for Tube 2

Step	Parameter	Temp [°C]	Cycles	Hold [mm:ss]
Hold	Enzyme Activation	95°	1	3:00
Cycling	Amplification	95°	40	00:10
		68°		00:30 (Acquiring to Cycling A on Green, Yellow, Red)

Fluorescence is detected in the **FAM**/Green, **HEX**/Yellow, and **Cy5**/Red channels at the end of the 2nd segment of the cycling step (68°C).

ADDITIONAL INSTRUMENT SETTINGS

The settings described in **Table 4** and **Table 5** are recommended for use with the Qiagen Rotor-Gene Q instrument. Perform gain optimization on a tube corresponding to No Template Control and set the instrument to perform optimization before first acquisition.

Table 4. Gain Optimization Settings for Tube 1

Channel	Min Reading	Max Reading	Min Gain	Max Gain
FAM/Green	70Fl	80Fl	-10	10
HEX/Yellow	70Fl	80Fl	-10	10
ROX/Orange	70Fl	80Fl	-10	10
Cy5/Red	70Fl	80Fl	-10	10

Table 5. Gain Optimization Settings for Tube 2

Channel	Min Reading	Max Reading	Min Gain	Max Gain
FAM/Green	70Fl	80Fl	-10	10
HEX/Yellow	70Fl	80Fl	-10	10
Cy5/Red	70Fl	80Fl	-10	10

DATA ANALYSIS

For Tube 1, fluorescent signal intensity is detected in four channels:

- The signal for *Atopobium vaginae* amplification is detected in the **FAM**/Green channel.
- The signal for *Gardnerella vaginalis* amplification is detected in the **HEX**/Yellow channel.
- The signal for *Lactobacillus spp.,* amplification is detected in the **ROX**/Orange channel.
- The signal for Internal Control amplification is detected in the **Cy5**/Red channel.

For Tube 2, fluorescent signal intensity is detected in three channels:

- The signal for *Megasphaera spp.* amplification is detected in the **FAM**/Green channel.
- The signal for BVAB2 amplification is detected in the **HEX**/Yellow channel.
- The signal for Internal Control amplification is detected in the **Cy5**/Red channel.

Results are interpreted using the instrument software by determining the cycle number, **Ct**, corresponding to the point where fluorescence signal drops below a defined threshold.

Using the Rotor-Gene Q software, the data for each of the four channels must be analyzed separately. For all four channels convert the data to display on a linear scale, select “Dynamic Tube” and ensure that “Slope Correct” is OFF. The settings recommended in **Table 6** and **Table 7** can be used to generate **Ct** values.

Table 6. Recommended Data Analysis Parameters for Tube 1

Channel	Ignore First	Threshold
FAM/Green	10	-0.04
HEX/Yellow	10	-0.04
ROX/Orange	10	-0.04
Cy5/Red	10	-0.04

Table 7. Recommended Data Analysis Parameters for Tube 2

Channel	Ignore First	Threshold
FAM/Green	10	-0.04
HEX/Yellow	10	-0.04
Cy5/Red	10	-0.04

INTERPRETATION OF PCR RESULTS

- The generation of a **Ct** value using the above analysis parameters is indicative of a positive signal.
- The absence of a **Ct** value when the above analysis parameters are applied is indicative of a negative signal.
- The BV Controls 1 – 3 must produce a positive signal in all channels (see **Table 8**). If this condition is not met, the entire run is invalid and must be repeated.
- The No Template Control must produce negative signals in the green, yellow, and orange channel. If these conditions are not met, the entire run is invalid and must be repeated.
- For any individual samples of human origin there must be a positive signal in the red channel with **Ct** < 35. If this condition is not met, results for the individual sample are considered invalid and the sample should be rerun.

Table 8. Expected Results for Assay Controls

Tube	Channel	BV Control 1	BV Control 2	BV Control 3	No Template Control
Tube 1	**FAM**/Green (*A. vaginae)*	HIGH POS	MED POS	LOW POS	NEG
	HEX/Yellow (*G. vaginalis)*	HIGH POS	MED POS	LOW POS	NEG
	ROX/Orange (*Lactobacillus spp.)*	HIGH POS	MED POS	LOW POS	NEG
	Cy5/Red (Internal Control)	POS	POS	POS	NEG or Ct >35
Tube 2	**FAM**/Green (*Megasphaera spp.)*	HIGH POS	MED POS	LOW POS	NEG
	HEX/Yellow (BVAB2)	HIGH POS	MED POS	LOW POS	NEG
	Cy5/Red (Internal Control)	POS	POS	POS	NEG or Ct >35

BV CONTROLS – TARGET COPY NUMBER

BV Controls 1 – 3 contain the following copies of the targets per µL.

Channel	BV Control 1	BV Control 2	BV Control 3
A. vaginae	10^5	10^3	10
G. vaginalis	10^5	10^3	10
Lactobacillus spp.	10^5	10^3	10
Megasphaera spp.	10^5	10^3	10
BVAB2	10^5	10^3	10
Internal Control	POS	POS	POS

QUANTIFICATION METHOD

A standard curve can be generated for each channel utilizing the target copy number values provided for the BV Controls alongside the Ct values obtained for each by the PCR reaction. Under the “Samples” tab in the Rotor-Gene software the sample “Type” can be assigned. By setting the “Type” for a sample from the default “Unknown” to “Standard”, a value can be entered under “Given Concentration”. Once all three BV Controls are set as “Standards” and a concentration is provided, the software will automatically generate the standard curve and provide a calculated concentration for all the samples tested.

PERFORMANCE CHARACTERISTICS

Overall assay detection sensitivity will vary based on the methods used for nucleic acid extraction and should be verified independently.

Analytical sensitivity of the PCR reaction, based on results obtained using plasmid controls, is estimated to be in the range of 1 to 50 target copies per reaction.

FREQUENTLY ASKED QUESTIONS

Can this assay be run on an ABI 7500?

Most thermal cyclers can be used, provided the software is equipped to detect fluorescence decay. However, the PCR method profile and subsequent data analysis parameters may require instrument-specific modification. The software accompanying the ABI 7500 instrument is not capable of generating Ct values for a fluorescence quenching assay and although the assay can be run, data must be analyzed independently.

What is the composition of the BV Controls 1 – 3 and the No Template Control (NTC)?

The PCR controls supplied with the AMPIPROBE® Bacterial Vaginosis Assay Kit are composed of linearized plasmids containing the specific sequences targeted by the primers in this assay. The amount of target in each mixture is formulated to produce a positive result falling within the dynamic range of the assay when it is carried out according to the procedure described in this user manual.

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GLOBAL HEADQUARTERS
Enzo Life Sciences Inc.
10 Executive Boulevard
Farmingdale, NY 11735
Toll-Free:1.800.942.0430
Phone:631.694.7070
Fax: 631.694.7501
info-usa@enzolifesciences.com

EUROPE
Enzo Life Sciences (ELS) AG
Industriestrasse 17
CH-4415 Lausen
Switzerland
Phone:+41/0 61 926 89 89
Fax:+41/0 61 926 89 79
info-ch@enzolifesciences.com

For local distributors and detailed product information visit us online:
www.enzolifesciences.com