

1. Innocreate Bioscience® AHPND QD Kit* (96 rxn.)

Cat. No. :P01-01-03-96

For use with the *ABI PRISM® 7000, 7500, 7700 and 7900HT Sequence Detection Systems* (Applied Biosystems) for the quantitative detection of AHPND DNA.

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2. Materials and Equipment

Kit Contents

The **Innocreate Bioscience® AHPND QD Kit v1.0** contains reagents for 96 reactions.

Components are shown in the table below.

Cap Color	Component Name	Volume	Tube
Blue	QD reagent set (QD)	220 µl	1
Red	2X Reaction Master Mix (RM)	1100 µl	1
Yellow	AHPND QD Standard (AHPNDSTD) (10 ⁷ copies/µl)	100 µl	1
Green	10X LTE buffer	500 µl	1
Orange	Distilled Water DNase, RNase Free	1000 µl	2

Storage

Follow the guidelines below for storing the **AHPND QD Kit v1.0**:

- Upon receipt, store the QD reagent set at -20°C and protect from light.
- Store the AHPND QD Standard DNA at -20°C
- Store the 2X Reaction Master Mix at -20°C . and protect RM reagent from light. Excessive exposure to light may affect the fluorescent reference dye. When Master Mix thaw out, it can be stored and protected from light at 2~8°C for up to 2 months.
- Do not freeze-thaw **AHPND QD Kit** excess 5 times.

Equipment and Materials Not Included

The following tables include equipment and materials for using the **AHPND QD Kit v1.0**. Unless otherwise noted, many of the items listed are available from major laboratory suppliers (MLS).

Instruments	Source
Applied Biosystems 7900HT Fast Real-Time PCR System	Applied Biosystems
Applied Biosystems 7500 Real-Time PCR System	
Applied Biosystems 7700 Real-Time PCR System	
ABI PRISM® 7000 Sequence Detection System	

Desktop centrifuge with rotor for 2 ml reaction tubes	MLS
Bench top micro centrifuge (for spin down 0.2ml strips)	MLS
Vortex mixer	MLS
Pipettes (adjustable) L1000 / L200 / L20 / L10 / L2 / Multichannel L8-20 (RAININ)	MLS (RAININ LTS systems is recommend)

Attention: A valid calibration of the pure dyes (*Pure Spectra Component File*) and the background (*Background Component File*) is necessary when putting the instruments into operation.

Materials	Source
High-Throughput Setup	
MicroAmp® Optical 96-Well Reaction Plate with Barcode (20 plates)	Applied Biosystems (PN 4306737)
MicroAmp® Optical Adhesive Film (100 covers)	Applied Biosystems (PN 4311971)
Thermo-Fast® 96 PCR Detection Plate MkII (25 plates)	ABgene (AB-1400)
ABSOLUTE™ QPCR SEAL (50 sheets)	ABgene (AB-1170)
Mid-to-Low-Throughput Setup	
MicroAmp® 8-Tube Strip, 0.2 ml (125strips)	Applied Biosystems (N8010580)
MicroAmp® Optical 8-Cap Strip (300 strips)	Applied Biosystems (PN 4323032)
Strips of 8 Thermo-Tubes & Ultra Clear Cap Strips	ABgene (AB-1183)
Strips of 8 Thermo-Tubes & Domed Caps (0.2ml / 250 Strips)	ABgene (AB-0266)
Other Materials	
Disposable powder-free gloves	MLS
Pipet tips, aerosol resistant GP-L10F/GP-L250F/GP-L1000F	MLS (RAININ LTS Filter Tips is recommend)
RNase-free, sterile-filtered water	MLS
Sterile microcentrifuge tubes, 1.5ml / 2.0 ml	MLS

3. Preventing Contamination

Overview

PCR assays require special laboratory practices to avoid false positive amplifications (Kwok and Higuchi, 1989). The high sensitivity of these assays can lead to amplification of a single DNA molecule (Saiki *et al.*, 1985; Mullis and Faloona, 1987).

Preventing PCR Contamination

- If possible, maintain separate work areas, dedicated equipment, and supplies for:
 - Sample preparation
 - PCR setup
 - PCR amplification
 - Analysis of PCR products

Note: Rooms can be simulated using a clean bench or PCR bench available from major laboratory

suppliers.

- Do not bring amplified PCR products into the PCR setup area.
- Wear a clean lab coat (not previously worn while handling amplified PCR products or used during sample preparation) and clean gloves when preparing samples for PCR amplification.
- Change gloves whenever you suspect that they are contaminated and before leaving the work area.
- To avoid false positives due to cross contamination, do not include a positive control unless required. If a positive control is necessary, close all unknown sample tubes before pipetting the positive control.
- Open and close all sample tubes and reaction plates carefully. Try not to splash or spray PCR samples.
- Keep reactions and components capped as much as possible.
- Use positive-displacement pipets or aerosol-resistant pipet tips.
- Clean lab benches and equipment after use with freshly diluted 10% bleach solution.

IMPORTANT! To avoid false positives due to amplified material in your work area, do not open tubes after amplification.

4. Definitions of Terms

This protocol assumes these definitions:

- **Amplification** – The process of making copies of, and thereby increasing the amount of, a specific DNA sequence.
- **Negative control** – Monitors for contamination (unexpected amplification in the absence of a target). One negative control is required for each target assay.
- **Polymerase Chain Reaction (PCR)** – Technology used to amplify, or increase the amount of, a DNA sequence.
- **Positive control** – Monitors for the expected amplification of a target. Target signal not detected in a positive control well indicates a pipetting error or a problem with amplification. A positive control is optional for each target assay and is not recommended because it can cause cross-contamination.
- **Premix solution** – A solution you prepare that contains 2X Reaction Master Mix (RM) and QD reagent set (QD).
- **Primer** – A segment of DNA that is complementary to the target DNA sequence or Internal Positive Control DNA sequence. Needed to initiate amplification.
- **Probe** – A segment of DNA that is complementary to the target DNA sequence. The probe is labeled with a reporter dye. When the probe binds to the target, a reaction detected by the Sequence Detection System (SDS) or Real-Time PCR System indicates the presence of the target.
- **AHPND QD reagent set (QD)**– Target-specific reaction for the AHPND / Shrimp DNA detection assay. QD is used to prepare the premix solution. QD reagent contains specific

primers and probes for the target.

- **2X Reaction Master Mix (RM)** – A common reagent for all detection assays. The RM is used to prepare the premix solution. It contains the polymerase enzyme that initiates PCR in the presence of the necessary primers and DNA sample.
- **10X LTE Buffer** – A reagent for quantitative recovery of small amounts of nucleic acids in dilute solutions, use for dilution AHPND QD Standard.
- **Target** – The pathogen being tested.
- **Unknown sample** – A DNA sample that you are testing for the presence of one or more pathogens.

5. General Precautions

The user should always pay attention to the following:

- Use sterile pipette tips with filters.
- Store and extract positive material (specimens, controls and amplicons) separately from all other reagents and add it to the reaction mix in a spatially separated facility.
- Thaw all components thoroughly at room temperature before starting an assay.
- When thawed, mix the components and centrifuge briefly.
- Work quickly on ice or in a cooling block.

6. Principle of Real-Time PCR

Pathogen diagnosis by the polymerase chain reaction (PCR) is based on the amplification of specific regions of the pathogen genome. In real-time PCR the amplified product is detected via fluorescent dyes. These are usually linked to oligonucleotide probes which bind specifically to the amplified product. Monitoring the fluorescence intensities during the PCR run (i.e. in real-time) allows the detection and quantitation of the accumulating product without having to re-open the reaction tubes after the PCR run (Mackay, 2004).

7. Product Description

The **Inncreate Bioscience® AHPND QD Kit** constitutes a ready-to-use system for the detection of AHPND DNA using polymerase chain reaction (PCR) in the *ABI PRISM® 7000, 7300, 7500 and 7900 Sequence Detection System* (Applied Biosystems). The *AHPND QD reagent* contains reagents for the specific amplification of the AHPND genome. The amplicon is detected by measuring the fluorescence in the *ABI PRISM® SDS*. In addition, the **Inncreate Bioscience® AHPND QD Kit** contains a second amplification system to measure the quantity of shrimp genomic DNA.

8. Preparing the PCR

Prepare the number of required reaction tubes or a 96-well reaction plate for the scheduled reactions. When preparing the PCR reaction, please make sure that at least one Quantitation Standard per PCR run as well as one negative control (Water, PCR grade) are performed in parallel. To generate a standard curve, use all supplied AHPND QD Standard (AHPNDSTD) for each PCR run.

8.1 Sample DNA dilution

10x Dilute eluted DNA from DNA isolation to prevent PCR inhibition effect. Transfer 10 μ l of the sample DNA to a new tube containing 90 μ l of Nuclease free water. Briefly vortex the new tube to mix the contents. The sample DNA is now ready for PCR, use 2 μ l for each reaction.

8.2 AHPND QD Standard prepare

1X LTE buffer is as the diluent for the AHPND QD Standard (10^7 copies/ μ l) dilution. Add 10 μ l AHPND QD Standard into 90 μ l 1X LTE buffer. Mix well to be 10^6 copies/ μ l standard. Use the same ratio to dilute the standard to 10^5 - 10^1 copies/ μ l. At least 5 standard (10^6 - 10^2) are required for calibration curve. Use 2 μ l standard for each standard reaction.

8.3 Prepare the reaction mix

- Create the Premix Solution according to the following table:

Preparation of Premix solution	sample number	1	24*	96*
	QD reagent set (QD)	2 μ l	52.8 μ l	211.2 μ l
	2X Reaction Master Mix (RM)	10 μ l	264 μ l	1056 μ l
	Nuclease free water	6 μ l	158.4 μ l	633.6 μ l
	Total volumn	18 μ l	475.2 μ l	1900.8 μ l

* Includes 10% overage to compensate for pipetting errors.

Note: Use a new tip when pipetting QD / RM / Nuclease free water.

Note: Innocreate Bioscience recommends performing two or three replicates for each sample. At least one negative control is required per target organism tested. Add one 20 μ l reaction per positive control, negative control, or replicate.

- Transfer 18 μ l of Premix Solution into each well to be used, gently pipetting at the bottom of the well.

Note: Use a new tip for each Target Premix Solution.

- Transfer 2 μ l of diluted unknown sample or standard into each sample well.

Attention: Before each use, all reagents need to be thawed completely, mixed (by repeated up and down

pipetting or by quick vortexing) and centrifuged briefly.

- Close the reaction tubes with the corresponding caps or, alternatively, when using a 96-well reaction plate, with optical adhesive covers (*Optical Adhesive Covers*, Applied Biosystems).
- Centrifuge the reaction tubes (in a storage rack for PCR tubes) or the 96-well reaction plate in a centrifuge with a microtitre plate rotor for 30 seconds at 1,780 x g (3,000 rpm), in order to collect the prepared reaction volume in the bottom of the tube or well.

If such a centrifuge is not at your disposal, please make sure that both the Master Mix and the sample volume are pipetted to the bottom of the tubes or wells. Store the prepared reactions at +4°C until the *ABI PRISM® SDS* Instrument is programmed and subsequently transfer them into the instrument.

9. Programming the *ABI PRISM® SDS2.3*

9.1 Pre-Settings for Creating a New PCR Run

Select the item *New* from the *File* menu on the *SDS2.3* and programme the following initial settings for the new document. A backup template (*SDS Template [*sdt]*) is available from the *Template* list or by selection using the *Browse* function. Confirm your pre-settings (*OK*).

9.2 Creating/Selecting the Detectors

With the *SETUP* menu, assign the corresponding detector dyes to the file. The reporters listed in the following table are to be defined:

Detection	Reporter
AHPND QD PirB	FAM
AHPND QD Pm18s	VIC

To create these detectors, select the option *Tools* (bottom left of the *Detector Manager*) and subsequently the option *New*. For the detection of AHPND QD PirB define the reporter combination **FAM** in the new window. For the detection of the AHPND QD Pm18s, also select the combination **VIC**. By confirming the input data (*OK*), return to the *Detector Manager*. Mark the newly created detectors and transfer each selection to the *Well Inspector* by clicking on the option *Add to Plate Document*. Close the window (*Done*).

9.3 Assigning the Necessary Information to the Plate Positions

Open *Well Inspector* from the *View* menu to find the detectors selected. Mark the plate positions reserved for the detection of AHPND DNA. Assign the selected detectors to these positions by activating the *Use* option of both detectors, upon which a tick will

appear. For the denomination of each single reaction, select the corresponding position on the plate and enter the name (*Sample Name*). Please note that preparations with an identical *Sample Name* and an identical detector assignment will be identified as replicates by the software and will be averaged with respect to the quantified pathogen load. Then select the corresponding function (*Task*) for each sample type according to the following table:

Sample Type	Function (Task)	Concentration (Quantity)
Sample	Unknown	-
Non-template control	NTC	-
Standard	Standard	10^6 - 10^2

To generate a standard curve, use all supplied AHPND QD Standard (10^6 - 10^2) per PCR run and enter the corresponding concentrations for each standard. Please note that for a PCR run with the *AHPND QD Kit*, **ROX** has to be set as a passive reference. The equal distribution of the ROX dye in all PCR preparations of a lot by mixing of the *Reaction Mix* guarantees the recognition and calculation of tube-to-tube variations (fluorescence differences between several PCR preparations) by means of the *Sequence Detection Software* (normalization).

9.4 Creating the Temperature Profile

To create a temperature profile, switch from the *Setup* level to the *Instrument* level in the software. Use default setting of temperature and cycles (**50°C, 3 minutes ; 95°C, 10 minutes then 95°C, 15 seconds ; 60°C, 60 seconds 40 cycles**). Make sure that the reaction volume is set to 20 µl.

9.5 Saving the PCR Run

Save the settings (*Setup*) as a template in order to make use of them again later in a modified or unchanged form. By saving the settings as a *SDS Template (*.sdt)* in the *Template Directory*, this file may be selected directly in the *New Document* window from the *Template* drop-down list. Copies stored in other folders have to be opened via *Browse*. Before starting the PCR run, save it again as a *SDS Document (*.sds)*, in order to guarantee the saving of the data that will be collected during the course of the PCR.

9.6 Starting the PCR Run

Start the PCR run by selecting the option *Start* from the menu item *Instrument* or the field *Start* on the *Instrument* level.

10. Data Analysis

A valid calibration of the dyes (*Pure Spectra Component File*) and the background (*Background*

Component File) is necessary when putting the instruments into operation. These calibration files are required for the exact calculation of the results as follows:

All interfering signals generated by the instruments which influence the measurement are eliminated by the *Sequence Detection Software* of the *ABI PRISM® Sequence Detection Systems* by means of the *Background Component File*.

Furthermore, interferences appear during multicolor analyses between the emission spectra of the single fluorescence dyes. The software of the *ABI PRISM® SDS* compensates these interferences by calculations using the spectrum data of the individual dyes stored in the *Pure Spectra Component File*. The software uses the same file for the assignment of the fluorescence data comprising the entire measurable spectrum collected in the course of the PCR to the programmed detectors. The fluorescence data of the individual dyes are subsequently divided by the value obtained for the signal of the passive reference (ROX) in order to account for tube-to-tube variations (fluorescence differences between several PCR preparations). The thus normalized signals may then be evaluated by means of the *Amplification Plot*.

The calibration files used for the evaluation of a PCR run are automatically stored with the saving of the run. If no **calibration files** are installed, please create these files according to the instructions given in the *ABI PRISM® SDS User Guide/Manual*. If more than one Innocreate Bioscience QD system is integrated in your PCR run (**note temperature profile**), these assays should be analyzed separately. Samples with an identical *Sample Name* and an identical detector assignment will automatically be identified as replicates by the *SDS Software* and will be averaged with respect to the quantified pathogen load.

The following results are possible:

1. Both AHPND / Shrimp DNA fluorescence signal is detected.

The result of the analysis is positive: The sample contains AHPND DNA.

In the case of non-shrimp sample used, no Shrimp DNA signal can be detected.

2. No AHPND fluorescence signal is detected. At the same time, the fluorescence signal from the *Shrimp DNA* appears.

In the sample no AHPND DNA is detectable. It can be considered negative.

In the case of a negative AHPND PCR the detected signal of the *Shrimp DNA* rules out the possibility of PCR inhibition.

3. Neither a AHPND fluorescence signal nor a Shrimp DNA fluorescence signal is detected.

No diagnosis can be concluded.

Information regarding error.

11. Quantitation

The enclosed *Quantitation Standards* (AHPND QD Standard (10^6 - 10^2)) are treated as previously

purified samples and the same volume is used (2 µl). To generate a standard curve on an *ABI PRISM® Sequence Detection System*, all 5 *Quantitation Standards* should be used and defined as standards with specification of the corresponding concentrations. The import of standard curves from previous runs is not possible with the *ABI PRISM® 7000, 7300 and 7500 SDS* software.

The *Quantitation Standards* are defined as copies/µl. The following equation has to be applied to convert the values determined using the standard curve into copies/ng of sample material:

$$\text{AHPND QD Pm18s (ng)} = 10^{\left(\frac{\text{Pm18s Ct value} - 20.904}{-3.998} \right)}$$

$$\text{A. Result (copies/ng)} = \frac{\text{AHPND QD PirB Result (copies)}}{\text{AHPND QD Pm18s (ng)}}$$