DESCRIPTION

The **AnaPha MONODOSE dtec-qPCR** contains individual ready-to-use tubes containing all the components needed for ***Anaplasma phagocytophilum*** detection by using qPCR. *Anaplasma phagocytophilum* (formerly *Ehrlichia phagocytophilum*) is an intracellular, Gram-negative bacterium that belongs to Ehrlichiaceae family. It is a vector borne zoonotic disease whose morula can be visualized within neutrophils from the peripheral blood and synovial fluid. It causes anaplasmosis in sheep and cattle, also known as tick-borne fever and pasture fever, and also causes the human granulocytic anaplasmosis a zoonotic disease. *Anaplasma phagocytophilum* can cause lethargy, ataxia, loss of appetite, and weak or painful limbs. This organism causes lameness which can be confused with symptoms of Lyme disease, another tick-borne illness.

PRINCIPLE OF THE METHOD

Polymerase chain reaction (PCR) allows the amplification of a target region from a DNA template by using specific oligonucleotides. In real-time PCR (qPCR), the accumulating amplified product can be detected at each cycle with fluorescent dyes. This increasing signal allows to achieve sensitive detection and quantification of pathogens.

KIT CONTENT

TargetSpecies MONODOSE dtec-qPCR (INDIVIDUAL TUBES), contains a dehydrated mixture for qPCR amplification. 24 or 96 reactions

[OPTIONAL] Internal Control, if requested the MONODOSE tubes will include primers, probe, and DNA template to evaluate PCR inhibition.

DNase/RNase free water (GREEN CAP), 1.5 ml

Standard Template (RED CAP), dehydrated target copies for positive control.

Template buffer (BLACK CAP), exclusive for resuspension of the Standard Template. 150 µl

STORAGE CONDITIONS

All the components of **AnaPha MONODOSE dtec-qPCR** are stable at room temperature for transport. At arrival, if not immediately used, it should be stored at -20 ºC. Individual tests are stable for one year (see expiration date on the label).

For **Standard Template** (RED CAP) we recommend, once dissolved, store **in an exclusive box** at -20 ºC.

MATERIALS REQUIRED BUT NOT PROVIDED

- DNA isolation kit (*GPSpin* extraction/purification kits recommended)
- DNase/RNase free water (to prepare standard curve dilution)
- Micropipettes and sterile pipette tips with filters
- Low retention tubes certified as DNase/RNase free
- Vortex mixer and spinner centrifuge
- Cooling block
- Real-time PCR device

WARNINGS AND PRECAUTIONS

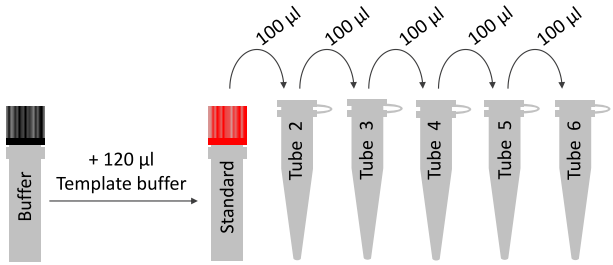
ⓘ For Research Use Only (RUO).
ⓘ To avoid possible misuse, carefully read the handbook.
ⓘ Proper training is recommended for correct operation of the kit.
ⓘ According to good laboratory practices, always wear a suitable lab coat, disposable gloves, and protective goggles.
ⓘ All the instruments used must been verified and calibrated according to the manufacturer's recommendations.
ⓘ Any kind of sample can be analysed with this assay after appropriate nucleic acid extraction.

General precautions

⚠ To prevent contamination of micropipettes, use sterile tips with filters.
⚠ Micropipettes used to dilute the Standard Template should not be used for other PCR reagents.
⚠ Extract, store and prepare positive materials (samples, positive controls and PCR products) in a separately laboratory environment.
⚠ To avoid cross-contamination with the positive control, pipette it after closing reaction tubes with negative control and samples.
⚠ The workflow in the laboratory should be unidirectional, from clean preamplification area to the amplification area.
⚠ Keep components refrigerated in a cooling block.
⚠ Protect the primer/probe from prolonged exposure to light.
⚠ After preparing MONODOSE tubes, run reactions immediately.

PREPARATION OF STANDARD CURVE DILUTION SERIES

1) Pipette 900 µl of **DNAse/RNAse free water** (not provided) into five tubes and label as 2 to 6
2) Pulse-spin the **Standard Template** (RED CAP), reconstitute with 120 µl of **Template buffer** (BLACK CAP) and vortex thoroughly, label as num. 1
3) Pipette 100 µl of diluted **Standard Template** (RED CAP), into tube 2
4) Vortex thoroughly and pulse-spin
5) Change tip and pipette 100 µl from tube 2 into tube 3
6) Vortex thoroughly and pulse-spin
7) Repeat steps 5 and 6 with the tubes 4 to 6 to complete serial dilution



Standard curve dilution series	copies/µl	copies in 5 µl
Standard Template (RED CAP)	2×10^5	10^6
Tube 2	2×10^4	10^5
Tube 3	2×10^3	10^4
Tube 4	2×10^2	10^3
Tube 5	2×10	10^2
Tube 6	2	10

Pipette 5 µl of template into each well for the standard curve according to your plate set-up. The final volume in each qPCR reaction well is 20 µl.

PROTOCOL & AMPLIFICATION REGIME

Add the desired volume of sample to each single tube ranging from 5 µl up to a maximum qPCR volume of 20 µl and, when needed, complete this final volume by adding DNase/RNase free water (GREEN CAP) (i.e., 7 µl sample + 13 µl water). Vortex thoroughly and pulse-spin. To determine the sample volume, please take into account the possible presence of inhibitors.

GPS™ reagents are **compatible with all real-time PCR thermal cyclers, glass capillary or plate based**. Plastic of the *Generic tube* is compatible with: StepOne™, StepOnePlus™, ABI 7500 Fast, LightCycler® 96, LightCycler® Nano, CFX96™, PikoReal™ 24-well, DNA Engine® systems, MiniOpticon™ 48-12 and Opticon® 2. For other devices, please, transfer the content of the MONODOSE (20 µl) to appropriate tubes.

Take into account that the fluorescent signal must be collected by using the FAM channel for the target. If the Internal Control is added use the HEX channel. Probe includes a Dark Quencher which can be set up as any non-fluorescent quencher (NFQ) in the software. The use of a passive dye as ROX is not required.

	Step	Time	Temperature
	Activation	2 min	95 ºC
40 Cycles	Denaturation	5 sec	95 ºC
	Hybridization / Extension and data collection[1]	20 sec	**60 ºC**

1 Fluorogenic signal should be collected during this step by using the **FAM** channel for the target and by using the **HEX** channel for the Internal Control.

RECOMMENDED REACTION CONTROLS

These qPCR reaction controls are recommended when considering the guidelines of ISO/IEC 17025 Standard. When setting-up your qPCR protocol, select the controls considered better suits your quality system.

Negative Control (Ctrl -): Add 20 µl of DNase/RNase free water (GREEN CAP) to one tube. Accordingly, this reaction should be negative. A positive result may be considered as a symptom of contamination in the water, making the test inconclusive. Water must be replaced.

Positive Control (Ctrl +): Prepare a standard curve dilution series as described above from the **Standard Template** (RED CAP). Add 5 µl of the Standard Template dilution (i.e., 2 x 10^2 copies/µl; Tube 4) to 15 µl of DNase/RNase free water (GREEN CAP). A positive result indicates that qPCR setup is correct and works. If negative, the test should be carefully repeated after checking the thermal protocol.

Sample Inhibition Control (sIC): We recommend to run reactions in parallel to test possible inhibition effects of compounds (inhibitors) present in the sample matrix. Simply, to a reaction tube, add a known amount of **Standard Template** (RED CAP) (i.e., 2 x 10^2 copies/µl) together with the sample.

Reagent	Volume
Standard Template dilution (i.e., 2 x 10^2 copies/µl)[1]	5 µl
DNase/RNase free water (GREEN CAP)	10 µl
Sample	5 µl
FINAL REACTION VOLUME	**20 µl**

1 Tube 4 of the curve dilutions series obtained from **Standard Template** (RED CAP).

An optimal result should show a positive signal, equal or higher (same or lower Ct) than these found for the Positive Control alone (tube 4, 10^3 copies). Inhibition may be total (negative result) or partial, observing a considerable increase in the Ct when compared to this of the Standard

Template dilution added. If inhibition is observed, a sample dilution to 1/10 may be recommended (if concentration is not close to detection limit). The sample inhibition control is external, allowing to check the inhibition on the main target of interest.

Extraction Negative Control (ExtCtrl -): Perform an extraction according to your extraction protocol without addition of sample. Add 5 µl of extraction negative sample to 15 µl of DNase/RNase free water (GREEN CAP). In this case, the test includes the reagents used in the extraction steps. If positive, when the **Negative Control** is negative, a contamination occurs during the extraction process. Extraction reagents must be discarded.

Extraction Positive Control (ExtCtrl +): Perform an extraction according to your extraction protocol adding the **Standard Template** (RED CAP), or nucleic acid material extracted from pure cultures into the first extraction buffer. The positive extraction control would include the effectiveness of the extraction method used. A positive result should be expected. If negative, extraction must be carefully repeated, or the extraction method replaced.

INTERPRETATION OF RESULTS

The linear regression obtained from the logarithm of the copy number versus Ct gives both constants Y-intercept and slope of the standard curve (equation 1). The number of copies in the sample can be calculated based on the regression (equation 2).

$$Ct = Y\ intercept + Slope\ x\ log(copy\ number) \quad (1)$$

$$Copy\ number = 10^{\frac{(Ct - Y\ intercept)}{Slope}} \quad (2)$$