MolliScience® ts-11 / MG304 DIVA qPCR Kit User Manual

1. General Information

- **Intended Use:** This kit is an *in vitro* molecular assay for the detection and differentiation of wild-type *Mycoplasma gallisepticum* and the ts-11 / MG304 vaccine strains (Vaxsafe® MG, Strain TS-11 and Vaxsafe® MG304; Bioproperties Pty. Ltd.) DNA. The kit distinguishes wild-type strains from vaccine strains using specific genetic markers, and is applicable directly on DNA samples of clinical samples (e.g. tissue, swabs, secretions, cell cultures, FTA card) and isolates. The test is for veterinary use only and should be performed by qualified laboratory personnel.
- **Principle of the Method:** The kit employs multiplex real-time PCR with fluorescent probes. Primers and probes target two pathogen-specific genes (i.e. wild-type and vaccine strain markers). During PCR amplification, probes labelled with distinct fluorophores (FAM for wild-type specific target, HEX for vaccine specific target) emit fluorescence proportional to the amount of amplified DNA. The real-time PCR instrument detects these signals cycle-by-cycle to determine the presence of each target gene.
- **Safety Notes:**
    - For ***in vitro*** use only. Do **not** ingest or pipette by mouth.
    - Treat all specimens as potentially infectious. Follow biosafety procedures.
    - Wear protective clothing, gloves, and eye protection when handling samples and reagents.
    - Use aerosol-resistant filter tips to prevent contamination. Change tips between samples.
    - Dispose of waste (biological and chemical) according to local regulations.
    - Refer to Safety Data Sheets (SDS) for information on hazardous components (e.g. polymerase).
    - Keep the kit components out of reach of children.

2. Kit Contents and Storage Conditions

Component	Cap Colour	Description	Quantity (per kit)	Storage
ts-11 / MG304 DIVA qPCR Reaction Mix 1	white	Liquid ready-to-use PCR mix containing buffer, dNTPs and polymerase.	2 × [600 µL] (100 rx kit)	–20 °C
ts-11 / MG304 DIVA qPCR Reaction Mix 2	yellow	Liquid ready-to-use PCR mix containing primers and probes (FAM, HEX).	1 × [500 µL] (100 rx kit)	–20 °C
ts-11 / MG304 DIVA qPCR Reaction Mix 3	blue	Nuclease-free water. Used as negative control (no-template control) also.	1 × 1 mL (100 rx kit)	–20 °C (or 2–8 °C)
ts-11 / MG304 DIVA qPCR Positive Control	red	Ready-to-use DNA control (wild-type and vaccine target).	1 × [50 µL] (100 rx kit)	–20 °C

- **Storage:**
  - For short-term storage (1-4 days) store all kit components at 2–8 °C.
  - For long-term storage (> 4days) store all kit components at **–20 °C**. The ts-11 / MG304 DIVA qPCR Reaction Mix 3 (Nuclease-free water) may alternatively be stored at 2–8 °C.
  - Avoid repeated freeze–thaw cycles: prepare single-use aliquots if needed.
  - Protect ts-11 / MG304 DIVA qPCR Reaction Mix 2 from light.
- **Shelf Life:**
  - Use kit components within the stated expiration date.
  - Do not mix reagents from different lots.
- **Handling:**
  - Thaw reagents on ice or at room temperature before use.
  - Vortex and briefly centrifuge reagents after thawing.

3. Additional Equipment and Reagents Required

- **Real-Time PCR Instrument:** Thermocycler capable of real-time fluorescence detection (e.g. Rotor-Gene, Bio-Rad CFX96, ABI 7500). Must detect the fluorophores used (FAM, HEX or equivalents).
- **Consumables:** PCR tubes or plates and optical caps/seals compatible with your instrument.
- **Pipettes and Tips:** Adjustable micropipettes (e.g. 2–20 µL, 20–200 µL) with sterile aerosol-resistant tips.
- **General Lab Equipment:** Microcentrifuge, vortex mixer, refrigeration (2–8 °C), freezer
  (–20 °C or colder).
- **DNA Extraction Supplies:** Kits or reagents (columns, magnetic beads, etc.) for nucleic acid purification from animal samples.
- **Computer/Software:** For PCR machine control and data analysis (if required by instrument).

4. Sample Collection and Preparation for DNA Extraction

- **Sample Types:** Suitable for a variety of veterinary samples, including DNA extracted from swabs (palatine cleft, trachea, air sac, cloaca, etc.), tissues (trachea, lung, air sac, genital tract, joint, egg, etc.), FTA cards, and cultured cells.
- **Collection Guidelines:** Use sterile techniques. Collect at least 0.5 mL of fluid or 1–2 g of tissue. Label samples clearly.
- **Handling:** Keep samples cold (2–8 °C) during transport. Process samples as soon as possible. For longer storage (>24 h), freeze at –20 °C or –80 °C. Avoid multiple freeze–thaw cycles.
- **Swabs:** Vortex swab in buffer (e.g. PBS) to release cells/virus. Centrifuge the eluate, remove supernatant, and proceed with DNA extraction.
- **Tissues:** Homogenize approximately 25–30 mg tissue in buffer (e.g. PBS). Centrifuge, remove supernatant, and proceed with DNA extraction.
- **Cell Cultures:** Pellet cells by centrifugation, remove supernatant, and proceed with DNA extraction.
- **Pooling (if applicable):** If pooling samples, combine a limited number (e.g. up to 5) of similar samples in a buffer. Document pooling scheme.
- **DNA Extraction:** Extract nucleic acids using a validated protocol that yields pure DNA free of inhibitors. Improper extraction can cause false negatives or inhibition. Keep extracted DNA at 2–8 °C short-term (days) or –20 °C long-term. Avoid repeated freeze–thaw of the extract.
- **Precautions:** Always include appropriate controls. Work in a designated area to avoid contamination. Change gloves frequently and use new tips between samples.

5. Reaction Setup and PCR Conditions

1. **Reagent Preparation:** Thaw ts-11 / MG304 DIVA qPCR Reaction Mix 1-3 and Positive Control. Vortex and spin down.
2. **Master Mix Preparation:**

Component	Volume for 1 sample (µl)	Volume for "n" sample (µl)
ts-11 / MG304 DIVA qPCR Reaction Mix 1	10	1.1 × 10 × n
ts-11 / MG304 DIVA qPCR Reaction Mix 2	4	1.1 × 4 × n
ts-11 / MG304 DIVA qPCR Reaction Mix 3	4	1.1 × 4 × n

3. **Master Mix Dispensing:** Label PCR tubes or plate wells. Dispense **18 µL** of Master Mix into each well.
4. **Add Samples/Controls:** Pipette carefully to avoid bubbles and cross-contamination. Add ts-11 / MG304 DIVA qPCR Positive Control last.
    a. Add **2 µL** of Nuclease-Free Water (ts-11 / MG304 DIVA qPCR Reaction Mix 3) to the NTC well (negative control).
    b. Add **2 µL** of each DNA extract to the respective sample wells.
    c. Add **2 µL** of ts-11 / MG304 DIVA qPCR Positive Control to the designated control well.
5. **Sealing and Centrifugation:** Seal the plate or tubes with optically clear caps or adhesive film. Briefly centrifuge the plate/tubes (e.g. 1–2 minutes at 1000–2000 ×g) to collect liquid at the bottom and remove bubbles.
6. **Thermal Cycling:** Place the plate in the real-time PCR instrument. Use the following cycling program:

Step	Temperature	Time	Cycles
1. Enzyme Activation	95 °C	2 min	1
2. Denaturation	95 °C	5 sec	40
3. Annealing/Extension	60 °C	20 sec* (with fluorescence read)	

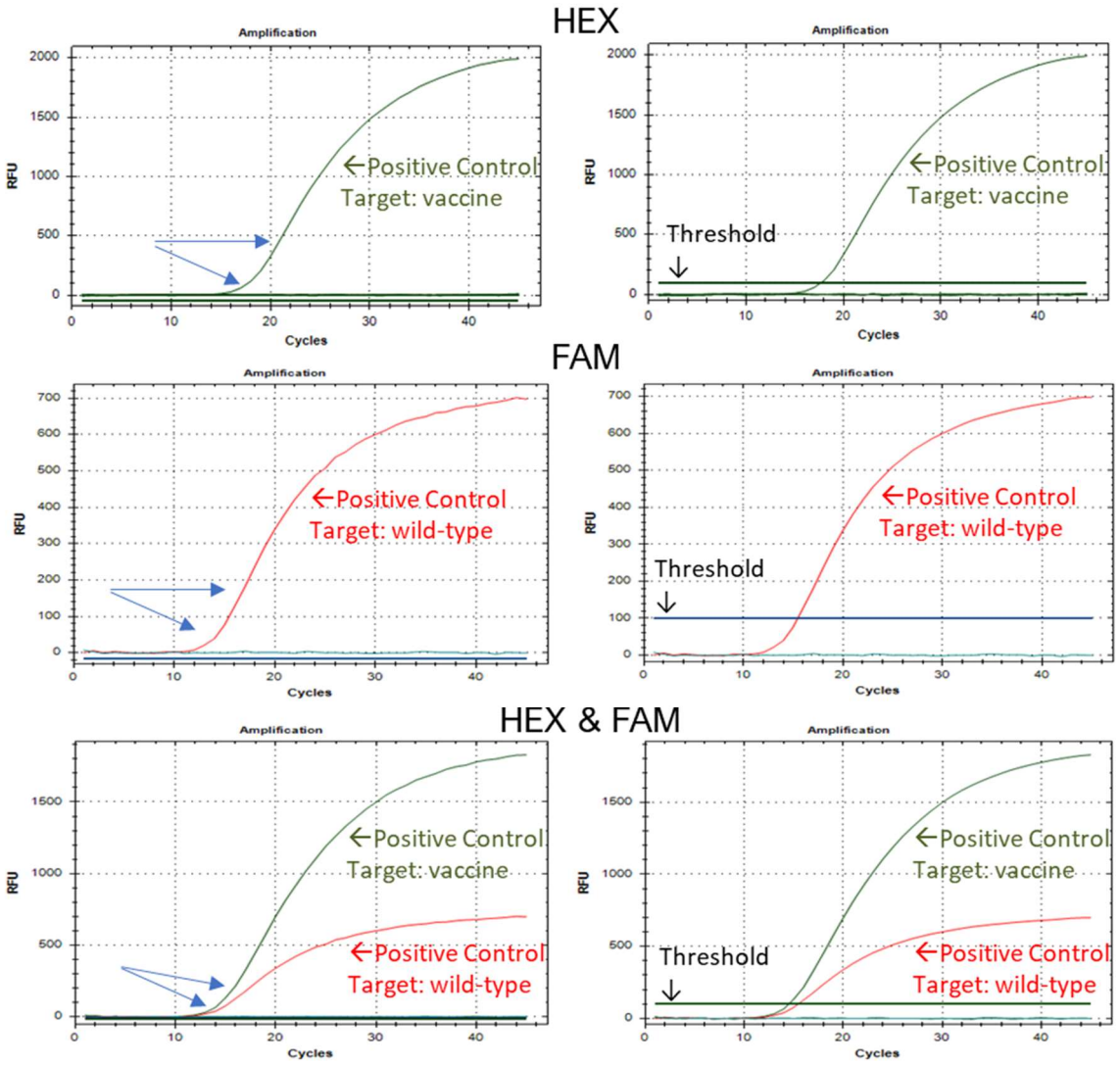
Data Acquisition: Detect fluorescence in FAM and HEX channels during each anneal/extension step.

Adjust annealing/extension time to suit your instrument's ramp rate.

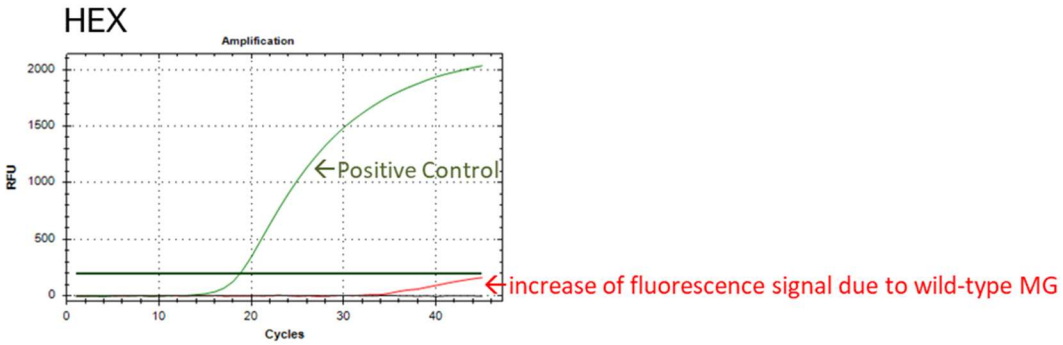
7. **Instrument Settings**:
    - Select the correct dye for each target channel.
    - If using an ABI instrument, disable the passive reference dye (ROX).
    - Use instrument default ramp speeds.
    - Ensure the lid is heated (if available) to prevent condensation.
8. **Precautions**: Avoid opening plate/tubes after PCR. Clean pipettes and work area regularly to prevent amplicon contamination.

7. Control Reactions and Interpretation – Validation of results

- **Positive Control:** Includes positive controls for both targets (mixed sequence of wild-type and vaccine strain; ts-11 / MG304 DIVA qPCR Positive Control). Include Positive Control in each PCR run. Positive Control should show a clear amplification curve in both FAM and HEX channels.
- **Negative Control:** Include a no-template control (NTC) in every run (ts-11 / MG304 DIVA qPCR Reaction Mix 3). It should show **no amplification** in the target channels.
- **Threshold Setting:**
  - Set the fluorescence threshold above the background (baseline) level. Ideally, set thresholds in the log-linear phase for each channel (FAM, HEX) (e.g. between the blue arrows in the picture below).



- In case of samples with a high amount of wild-type MG DNA load (as could occur in the case of the provided ts-11 / MG304 DIVA qPCR Positive Control), a minimal increase in background fluorescence in the HEX-channel might be determined, depending on the Real-Time PCR thermal cycler used. Set the threshold for the HEX-channel above the background fluorescence of the Positive Control (as shown in the picture below).



- **Run Validation:** A run is considered valid only if:
  - ts-11 / MG304 DIVA qPCR Positive Control amplifies with Ct values within the expected range (as defined during kit validation and presented in the certificate included in the kit).
  - Negative Control (ts-11 / MG304 DIVA qPCR Reaction Mix 3) shows no target amplification.
- **Interpretation Matrix:** Results for each sample are determined by the presence (+) or absence (–) of amplification in the FAM and HEX channels.

Control	FAM (Target wild-type)	HEX (Target vaccine)	Interpretation
Negative	–	–	Negative (no target detected)
Positive	+	+	Positive for both wild-type MG and the ts-11 / MG304 vaccines

'+' indicates amplification detected (Ct within cutoff, ≤40), '–' indicates no amplification. Results should be reported qualitatively (Positive/Negative) for each target per sample.

8. Data Analysis and Result Interpretation

- **Amplification Curves:** After the run, examine the amplification plots. Verify that amplification curves show a clear exponential phase.
- **Threshold Setting:** Set the fluorescence threshold above the background (baseline) level. Ideally, set thresholds in the log-linear phase for each channel (FAM, HEX).
- **Cycle Threshold (Ct):** Record the Ct value where each curve crosses the threshold. A lower Ct indicates a higher initial copy number.
- **Control Checks:** see section 7.
- **Result Criteria:**
    - A sample is **positive** for a target if a sigmoidal amplification curve is observed in the corresponding channel with Ct below the cut-off (< 40).
    - A sample is **negative** if no amplification occurs in target channels (Ct undetermined).
    - If **both** target channels amplify, report the sample as positive for both targets.
    - **Interpretation Matrix:** Results for each sample are determined by the presence (+) or absence (–) of amplification in the FAM and HEX channels.

Channel	Negative Control	Positive Control	Sample	Interpretation
FAM	-	+	+	Controls worked. The sample contains wild-type MG only.
HEX	-	+	-	
FAM	-	+	-	Controls worked. The sample contains ts-11 / MG304 only.
HEX	-	+	+	
FAM	-	+	+	Controls worked. The sample contains both wild-type MG and ts-11 / MG304.
HEX	-	+	+	
FAM	-	+/-	+/-	Problem with Amplification. Repeat run after Troubleshooting.
HEX	-	-	-	
FAM	-	-	-	
HEX	-	+/-	+/-	
FAM	+	+	+/-	Possible contamination. Repeat run after Troubleshooting.
HEX	-/+	+	+/-	
FAM	-/+	+	+/-	
HEX	+	+	+/-	

'+' indicates amplification detected (Ct within cutoff, ≤40), '–' indicates no amplification. Results should be reported qualitatively (Positive/Negative) for each target per sample.

- **Documentation:** Record all Ct values, interpretation, and any anomalies. Maintain records per laboratory quality procedures.

9. Troubleshooting Guide

Issue	Possible Cause	Recommended Action
No amplification in *any* wells	Reagents degraded; PCR setup error; instrument failure.	Check that the reagents were thawed and mixed. Verify pipetting of Reaction Mixes. Ensure the PCR lid and block are heating. Repeat with fresh reagents.
ts-11 / MG304 DIVA qPCR Positive Control fails (no signal)	Incorrect setup; degraded ts-11 / MG304 DIVA qPCR Positive Control DNA; PCR failure.	Check pipetting. Use a fresh ts-11 / MG304 DIVA qPCR Positive Control aliquot. Confirm cycling parameters.
Negative Control shows amplification	Contamination of reagents or consumables.	Replace reagents and tips. Clean workspace with bleach or DNA decontaminant. Prepare reactions in a contamination-free area.
Weak target signal in the expected positive sample	Low template amount or partial degradation; PCR inhibition.	Check extraction quality. Dilute the DNA extract to reduce inhibitors and re-test.
High variability between replicates	Pipetting error or uneven mixing.	Ensure thorough mixing of master mix and samples. Use calibrated pipettes. Perform all replicates in the same PCR run.
Late amplification in all wells	Non-specific amplification or low-level contamination.	If Ct values are beyond the threshold (≥40) and curves are not true sigmoids, consider them negative. Improve plate sealing and mix quality.
Unexpected amplification curve shapes	Instrument software settings or primer-dimer formation.	Check data analysis settings (baseline, threshold). Verify primer/probe integrity. Repeat PCR with fresh reagents.

If problems persist, consult technical support with detailed run data. Always include run sheet, Ct values, and instrument information when seeking help.

10. Technical Specifications

- **Reaction Volume:** 20 µL total (18 µL Master Mix + 2 µL DNA/sample).
- **Contents of Reaction Mix:** Hot-start DNA polymerase, dNTPs, $MgCl_2$, primers, probes.
- **Fluorophores:** FAM (ex=495 nm/em=520 nm) for Target wild-type, HEX/VIC (538/554 nm) for Target vaccine.
- **Cycling Conditions:** Total run time ~1 hours. See Section 5. for recommended profile.
- **Analytical Sensitivity:** Detects 1–10 copies of target DNA per reaction (as determined during validation, see in certificate in each kit).
- **Specificity:** Primers and probes are specific to *Mycoplasma gallisepticum* wild-type and ts-11 / MG304 vaccine strains; no cross-reactivity observed with closely related organisms or other bacteria commonly infecting birds (for details see validation studies).
- **Instrumentation:** Validated on BioRad CFX96 platform.
- **Storage Temperature:** –20 °C (long term). Thawed reagents should be kept on ice or at 2–8 °C during use (up to 4 days).
- **Shelf Life:** 12 months from manufacture when stored as directed. See lot label for expiration date.
- **Regulatory Status:** Not for human diagnostic use. For veterinary use only. For *in vitro* use only.
- **Validation report:** Validation steps are detailed for quality control regarding specificity, sensitivity, repeatability and reliability of the assays. Available upon request (Email: **info@molliscience.com**).

11. Manufacturer and Contact Information

- **Manufacturer:**
  MolliScience Kft., Március 15. utca 1., 2051 Biatorbágy, Hungary, EU.
- **Website:** www.molliscience.com
- **Technical Support:** Email: **info@molliscience.com**
- **Sales Inquiries:** Email: **info@molliscience.com**
- **Trademark: MolliScience®** is a registered trademark.

Please consult the company catalog or website for the latest information on compatible products and ordering details.