mir | detect BIOTRACING

CE 2797

IVD

Instructions for use

M371-Test

In vitro diagnostic medical device – for use by professional users only

REF **MCS0105**

REF **MCS0115HT**

Please read these instructions for use carefully before using the test, and follow them carefully in order to ensure the reliability of the test results.

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mir|detect GmbH, Fischkai 1,
27572 Bremerhaven, Germany
www.mirdetect.de

Contents

1. Name and intended use of the product

The M371-Test is an VO (EU) 2017/746 IVDR certified *in vitro* diagnostic medical device based on the measurement of the relative quantification (RQ) of the tumor marker miR-371a-3p. For this purpose, miR-371a-3p and an endogenous control are quantified in 200 µl of blood serum using qPCR.

The M371-Test is a non-automated test with a qualitative interpretation of results that detects the presence of testicular germ cell tumors (type II Germ cell neoplasia in situ derived testicular germ cell tumor (GCNis-derived TGCT)) and can be used for diagnosis and follow-up monitoring of this tumor by expert users. The test population includes male adult patients with suspected or confirmed testicular germ cell tumor (type II, GCNis-derived TGCT). The test result cannot be used as the sole primary diagnosis of a testicular germ cell tumor or to detect recurrence. Any positive M371-Test should be confirmed by an appropriate clinical diagnostic procedure.

2. Technological basics of the test procedure

The M371-Test kit contains all reagents necessary to perform the blood test for the detection of germ cell tumors of the testis from already extracted miRNA. The optional M371-Test evaluation file is provided for the evaluation of the samples.

The detection method is based on the fluorescence-based detection of the microRNA (miRNA) miR-371a-3p by quantitative real-time PCR. In order to measure this tumor marker, RNA, including miRNA, must be isolated from the patient sample (serum). The reagents for this first isolation step are **not** included in the kit.

In the next step, the tumor marker miR-371a-3p as well as an additional miRNA that serves as an endogenous control (from here on: reference miR) are transcribed into cDNA using specific primers. In the following pre-amplification step, the cDNA is amplified in a PCR. Subsequently, the relative abundance of the tumor marker miR-371a-3p is determined by quantitative PCR and normalised via the reference miR. The earlier a fluorescence signal can be detected during qPCR, the more molecules of the tumor marker or the reference miR were present in the sample. These values are expressed as "Ct" values. The relative quantification (RQ) of miR-371a-3p is calculated according to the ΔΔCt method (Livak & Schmittgen, 2001) by the reference miR and a fixed value (calibrator). The ΔCt value is first calculated for each sample: ΔCt = Ct (miR-371a-3p) − Ct (reference miR). The difference between the ΔCt value of the sample and the ΔCt value of the calibrator, whose relative expression of miR-371a-3p is set equal to 1, is then calculated: ΔΔCt = ΔCt (sample) − ΔCt (calibrator). The relative expression is calculated using the following formula: $RQ = 2^{-\Delta\Delta Ct}$. The RQ value is a multiple of the expression of the calibrator. The optional evaluation file ("M371-Test Evaluation File", see Chapter 3.4. Optional accessories for the M371-Test) performs these calculations after the measured Ct values have been correctly transferred.

The results of the M371-Test must be evaluated according to the clinical scenario depending on whether the test is used to make a primary diagnosis or to detect recurrences in the follow-up of testicular tumor patients (Table 1). As part of a primary diagnosis, a clear statement for samples with an RQ between 5 and 10 is not possible (INDETERMINATE) because this is the range of the limit of quantification of the test. In this case, another M371-Test should be carried out with a fresh sample after a few weeks.

Table 1: Interpretation of M371-Test results depending on the clinical scenario.

Primary diagnosis (detection of primary tumors)	
RQ < 5	negative, low tumor probability
5 ≤ RQ < 10	Indeterminate (INDETERMINATE), repeat after a few weeks
RQ ≥ 10	positive, high tumor probability
Follow-up care (detection of recurrences)	
RQ < 15	negative, low tumor probability
RQ ≥ 15	positive, high tumor probability

For further explanation of the scientific evidence, see Chapter “13. Specific performance data” of these instructions for use.

Each run is carried out with a negative control (NC). For evaluation and validity of controls, see Chapter “10. Result analysis” of these instructions for use.

3. Reagents included in the kit

3.1. Components

The M371-Test is offered in two versions (item number MCS0105 and item number MCS0115HT).

Item number MCS0105 – contains reagents for five patient samples and five negative controls. The user can measure each sample individually with a negative control (Table 2).

CAUTION: The negative control in pre-amplification and qPCR is measured only **once**.

Table 2: Contents of the M371-Test kit MCS0105.

Reagent	**Container**	**Volume (µl)**
cDNA Solution (black)	1 tube	**135**
Reverse Transcriptase (yellow)	1 tube	**19.68**
RNase Inhibitor (transparent)	1 tube	**3.74**
PreAmp Solution (green)	1 tube	**418**
Target Solution (blue)	1 tube	**410**
Control Solution (violet)	1 tube	**410**
PCR-grade water (white)	1 tube	**1000**

Item number MCS0115HT – contains reagents for 15 patient samples and one negative control. The user must measure all samples in **one** run (Table 3).

Table 3: Contents of the M371-Test kit MCS0115HT.

Reagent	**Container**	**Volume (µl)**
cDNA Solution (black)	1 tube	**153**
Reverse Transcriptase (yellow)	1 tube	**22.30**
RNase Inhibitor (transparent)	1 tube	**4.24**
PreAmp Solution (green)	1 tube	**786**
Target Solution (blue)	1 tube	**770**
Control Solution (violet)	1 tube	**770**
PCR-grade water (white)	1 tube	**1000**

3.2. Reactive components of the M371-Test

cDNA Solution (black)

- miRNA-specific stem-loop primer for the target and reference miRNA

Reverse Transcriptase (yellow)

- Reverse transcriptase

PreAmp Solution (green)

- miRNA-specific primers for the target and reference miRNA
- DNA Polymerase

Target Solution (blue)

- miRNA-specific primers and probes for the target miRNA
- DNA Polymerase

Control Solution (violet)

- miRNA-specific primers and probes for the reference miRNA
- DNA Polymerase

3.3. Information and documents for the M371-Test

The instructions for use, safety data sheets, and video tutorials for carrying out the test are available on the mirdetect website at https://www.mirdetect.de/download. When revised instructions for use are published, customers are notified of the new version by email.

3.4. Optional accessories for the M371-Test

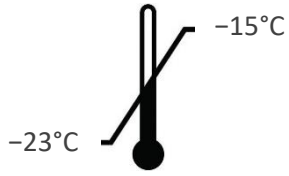
The M371-Test evaluation file (available only in English) is an optional spreadsheet software with stored formulas for the evaluation of samples and is transmitted in electronic form (via email). Updated versions are also sent by email.

3.5. Hazardous substances and animal components in the M371-Test kit

The components PreAmp Solution, Target Solution, and Control Solution contain formamide in low concentrations. For more detailed information on the concentrations, the safety data sheet for the M371-Test can be consulted (https://www.mirdetect.de/download).
One raw material used in the production of Target Solution and Control Solution contains animal gelatine. The manufacturer of the raw material assures that the risk of BSE/TSE contamination is negligible. In addition, the risk is minimised by using protective clothing (gloves, lab coat and safety goggles, see Chapter “6. Precautions”) and working under a PCR workbench whilst performing the M371-Test. The M371-Test may be carried out only by specialised users.

4. Transport, storage, and stability

The M371-Test is shipped < 0°C via express shipping. **In the event of transport damage, please contact both the transport company and mir|detect GmbH or Gold Standard Diagnostics Frankfurt GmbH**. The contact details are given in Chapter "17. Information for the purchaser". Damaged reagent tubes should not be used but rather disposed of immediately. Components of different kit lots should not be mixed together.



Store all reagents in the kit at −23°C to −15°C before and after opening for the first time. Protect the Target Solution (blue) and Control Solution (violet) from light. Each component can be defrosted and refrozen up to eight times.

If the storage conditions are complied with, the kit can be used until the expiry date indicated on the outside of the kit (maximum possible shelf life: 10 months). Do not use materials after the expiry date.

5. Additionally required equipment

5.1. General laboratory equipment

The following laboratory equipment is required to perform the M371-Test.

- Optional accessory: M371-Test Evaluation file software*
- PCR workbench
- Standard PCR instrument
- Cooling block for the reaction vessels used
- Vortex mixer
- Pipette with variable volume in suitable sizes
- Optional: Electronic dispenser
- Bench-top centrifuge with a rotor for 0.2/1.5 ml reaction tubes
- Plate centrifuge for PCR plates
- Real-time PCR instrument**

* The evaluation file for the M371-Test was validated with Microsoft Excel for Microsoft 365 MSO.
** The M371-Test was validated with the following real-time PCR cyclers:

- LightCycler® 480 II qPCR instrument (Roche Diagnostics) with 96-well heating block and software version 1.5.x
- QuantStudio™ 5 (Thermo Fisher Scientific) with 96-well heating block and "Design and Analysis" software, Version 1.4.x
- AriaDx (Agilent) with software version 2.0

5.2. General consumables and reagents

All consumables used should be made of polypropylene and must be free of RNases, DNases, DNA, and PCR inhibitors.

- Blood collection tube*
- Cryotube, self-standing
- miRNA extraction kit**
- 1.5 ml reaction tubes with conical bottom and safety lid (PP)
- 0.2 ml PCR reaction tubes (e.g. 8-tube strips)
- Pipette tips with filter
- Optional: Attachment for electronic dispenser
- PCR plates with adhesive foil
- Applicator for the application of adhesive foils

* necessary for serum collection. Recommended with Sarstedt AG & Co. KG S-Monovette® Serum Gel (7.5 ml or 9 ml Z-Gel); more detailed information on serum collection in Chapter "8. Sample collection and sample processing".
** necessary for the extraction of miRNA. Recommended assay is QIAGEN GmbH miRNeasy Serum/Plasma Kit.

5.3. Equipment requirements

The installation, calibration, functional qualification, and maintenance of all equipment used must be carried out according to the manufacturer's instructions and is the responsibility of the user of the test. The user is also responsible for establishing appropriate quality control procedures.

6. Precautions

The professional user is responsible for complying with applicable laboratory regulations. Always wear a suitable lab coat, disposable gloves, and protective goggles when working with chemicals.

6.1. Precautions in the laboratory

Regulations such as DIN EN ISO 17025 or DIN EN ISO 15189 should be adhered to in order to avoid the risk of cross-contamination of patient samples before, during, and after RNA extraction. Prevent the introduction of nucleases into the samples during extraction. Use only disposable pipette tips with filters in order to avoid cross-contamination between patient samples.

Measurement results can be influenced by strongly increased outside temperatures. Always store reagents and samples outside freezers in cooling blocks.

The reagents for qPCR (Control Solution (violet) and Target Solution (blue)) are light-sensitive and must therefore be stored away from light. Excessive exposure to light can affect the fluorescent probes.

The reagents of the M371-Test can be thawed up to eight times. Furthermore, the reagents should not be reused.

The M371-Test may be performed only by professionals familiar with methods of serum collection, RNA extraction, and qPCR.

6.2. Precautions to protect against infection

Human blood and serum samples tested with this test should always be treated as potentially infectious, and all precautions as required by the Microbiological and Biological Safety Directive for Laboratories, "Directive 2000/54/EC on the protection of workers from risks related to exposure to biological agents at work" or other biosafety regulations should be observed.

6.3. Reporting of events related to the product

Any serious incidents or events related to the product must be reported immediately to mir|detect GmbH (info@mirdetect.de) and the relevant authorities. Please do not make any medically relevant decisions without first contacting a health professional.

6.4. Disposal of working materials and reagents

All reagents of the M371-Test are not harmful to health. Expired reagents or empty reagent containers can be disposed of in the residual waste. Local regulations must be observed. Please **never remove the foil from used qPCR plates** and ensure that they are disposed of without damage.

For the handling of serum samples and their disposal or the working materials and reagents used for RNA extraction, please carefully read the instructions for use of the corresponding kits, and strictly adhere to them.

7. Quality control

According to the ISO 13485 certified quality management system of mir|detect GmbH, each batch of the M371-Test is tested against predetermined specifications in order to ensure consistent product quality. This keeps batch-to-batch variability low. Batch certificates are available from the manufacturer upon request.

8. Sample collection and sample processing

8.1. Blood collection and storage

Blood collection should be performed by qualified personnel in order to reduce associated risks to the patient. Subsequent blood storage and serum collection should be performed as described below.

- S-Monovette® serum gel tubes (Sarstedt AG & Co. KG) should be used for blood collection according to the manufacturer's instructions. Do not use plasma, EDTA, heparin, or PAXgene tubes.
- The serum should be separated from the blood cell components as soon as possible after blood collection (see 8.2. Serum collection, storage, and transport).
- **Whole blood samples may not be frozen** because this leads to haemolysis.

8.2. Serum collection, storage, and transport

- Invert the blood in the blood tube a few times and incubate upright for 30 min. at ambient temperature (15–25°C).
- Centrifuge the blood tube at 2500 *g* for 10 min.
- Carefully remove the blood tubes from the centrifuge.
- Pipette serum into a labelled cryo tube. Approximately 3–5 ml of serum should be obtained from 10 ml of whole blood.
- The serum can be stored for up to 6 h at 2–8°C if the RNA extraction is performed on the same day.
- For longer-term storage, aliquot the serum and store it at −20°C or −80°C.
- The serum should be transported in a suitable container, frozen. Stability can be maintained for the following duration:
    - 90 h at < −1°C
    - 16 days at < −20°C

8.3. Precautions for serum collection

If the serum has a conspicuous red colour, a photometric measurement at an absorbance of 414 nm is recommended. A value above 0.3 indicates a possibly problematic degree of haemolysis, which negatively influences the measurement result of the M371-Test (Myklebust *et al*., 2019). In this case, a new blood sample collection is advisable, and the haemolytic serum should be discarded.

A low Ct value (< 12) of the reference miR can also indicate the presence of haemolysis and falsify the result (see chapter “10.2.2. Reference miR ”).

If there is any indication that the serum is particularly fatty, let it sit at ambient temperature for a while. This forms a layer of fat, which can then be carefully removed.

Make sure that the layer of buffy coat (leucocyte film) above the red blood cells after the centrifugation step is not destroyed or co-transferred. This step is particularly important because a carry-over is the largest possible source of contamination with cellular miRNA or RNA.

8.4. miRNA extraction

Materials for the extraction of RNA or miRNA from patient serum are not included in the M371-Test.

To avoid degradation of the sample material during RNA extraction, the use of RNase-, DNase-, and DNA-free work equipment and personal protective equipment must be ensured. In addition, cross-contamination between patient samples must be avoided.

Attention: Avoid prolonged incubations and multiple thawing because this may lead to degradation.

The RNA extraction is performed according to the corresponding instructions for use. mir|detect GmbH recommends performing the RNA extraction from **200 µl of serum**. To ensure consistently efficient extraction, strictly follow the manufacturer’s instructions for the extraction kit.

- The extracted miRNA can be used directly to perform the M371-Test.
- The extracted miRNA should be stored at −20°C or −80°C.
- **Attention:** Repeated freeze-thaw cycles of miRNA should be avoided because they can lead to degradation!

9. Performance of M371-Test

All reagents of the M371-Test kit are “ready-to-use” and can directly be used to perform the test.

9.1. General performance of the test

Before using the M371-Test for the first time, it is recommended to perform a trial run with known samples. For support and advice, please contact **mir|detect GmbH** (contact form via https://www.mirdetect.de/Service/) or **Gold Standard Diagnostics Frankfurt GmbH** (17.3. Distributor).

For the monitoring and equivalent performance of the M371-Test in all laboratories, mir|detect GmbH recommends the participation in regular interlaboratory comparisons (contact form via https://www.mirdetect.de/Service/).

A negative control (NC) from PCR-grade water must be processed in each run in order to confirm the validity. Here, the control is transcribed in the cDNA synthesis. However, in contrast to a patient sample, analysed only as a single replicate in the final miR-371a-3p and reference miR measurement.

Important instructions:

- The components Reverse Transcriptase (yellow) and RNase Inhibitor (transparent) should **not** be mixed with a vortex mixer. Instead, flick the tube with your finger. Mix the remaining components on a vortex mixer for approx. 3 s at approx. 2,800 rpm before use in order to ensure a homogeneous solution.
- Centrifuge all solutions in the kit at 2,000 *g* for approx. 3 s before use in order to remove drops from the lid.
- Remove all kit solutions from their storage conditions only for performing the M371-Test. Use the cDNA synthesis master mix (MM) directly after it has been prepared. After use, all solutions must be refrozen immediately or empty containers disposed of.
- It is recommended to use appropriate cooling racks for all reaction vessels, including the 8-tube strips and the 96-well plate. These facilitate handling and ensure continuous cooling of the reagents.
- The temperature programs (Tables 6, 8, and 9) specify the conditions required for the respective reactions. In addition, the heated lid must be activated (recommended: 105°C). Please refer to the operating instructions for your device.

9.2. Performance of the cDNA synthesis

- Thaw cDNA Solution (black) and PCR-grade water (white) at ambient temperature or in the refrigerator.
- Mix the cDNA Solution on the vortex mixer for approx. 3 s, centrifuge, and store in the cooling block.
- Mix Reverse Transcriptase (yellow) and RNase Inhibitor (clear) by flicking (do not vortex), centrifuge, and store in cooling block.

Carry out the next steps under a clean PCR bench.

- Pipette the master mix (MM) for the cDNA synthesis from cDNA Solution, Reverse Transcriptase, and RNase Inhibitor together in a suitable reaction vessel according to the number of samples required. Note the ratio from Table 4.
- Mix master mix (MM) by flicking or pipetting up and down several times and centrifuge. Store master mix (MM) in the cooling block.
- Pipette 9 µl of the cDNA synthesis master mix (MM) per patient sample and control into an 8-tube strip (Table 5).
- Add 6 µl sample or control each.

Table 4: Pipetting scheme for the preparation of a cDNA synthesis master mix (MM)
Rxn = reactions

Master mix (MM) / **Reagent**	**Single sample**	**MM (2 samples)**
	1 Rxn (µl)	**3 rxn (including NC and 10% excess) (µl)**
cDNA Solution (black)	7.81	25.77
Reverse Transcriptase (yellow)	1.00	3.3
RNase Inhibitor (transparent)	0.19	0.63
Total volume	**9.00**	**29.7**

Table 5: Distribution of the cDNA synthesis master mix (MM) and samples into the PCR reaction tubes (8-tube strips). Illustration of the procedure for two samples and a negative control (NC).

PCR reaction vessels
MM + Sample 1
MM + Sample 2

MM + NC

- Mix and centrifuge cDNA synthesis preparations by flicking or pipetting up and down several times.
- Incubate cDNA synthesis mixtures for at least 5 minutes in a refrigerator or on ice at 4°C.
- Perform cDNA synthesis according to Table 6. Ensure that the heated lid is activated.

- Finished cDNA can be stored overnight in the refrigerator (4°C). Freeze at −20°C for longer storage.

Table 6: Parameters of the cDNA synthesis programme for a standard PCR instrument.

Target temperature (°C)	Duration (hh:mm:ss)	Segment
16	00:30:00	Annealing
42	00:30:00	Reverse transcription
85	00:05:00	Enzyme activation
≥ 4 to ≤ 10	∞	Cooling down

9.3. Performance of the pre-amplification

- Briefly thaw PreAmp Solution (green) at ambient temperature or in the refrigerator. Then mix for approx. 3 s on the vortex mixer, centrifuge off, and store in the cooling block.

Carry out the next steps under a clean PCR bench.

- For each patient sample, prepare **three** preparations of 16 µl PreAmp Solution in 8-tube strips, and add 4 µl each of the newly synthesised cDNA (Table 7).
- For the negative control, 16 µl of PreAmp Solution and 4 µl of cDNA preparation are sufficient.
- Mix and centrifuge the pre-amplification by flicking or pipetting up and down several times.
- Perform pre-amplification according to Table 8. Ensure that the heated lid is activated.
- Finished pre-amplicons can be stored overnight in the refrigerator (4°C). Freeze at −20°C for longer storage.

Table 7: Illustration of the performance of a pre-amplification for two samples and a negative control (NC).

PCR reaction vessels
Sample 1
Sample 1
Sample 1
Sample 2
Sample 2
Sample 2

NC

Table 8: Parameters of the pre-amplification program for a standard PCR instrument.

Cycles	**Target temperature (°C)**	**Duration (hh:mm:ss)**	**Segment**
1	95	00:01:00	Enzyme activation
15	95	00:00:15	Denaturing
	60	00:04:00	Annealing + elongation
1	≤ 10	∞	Cooling down

Note: It is important to ensure that the correct number of cycles is programmed because the input of cycles can vary in different PCR cycler systems (total number of cycles or number of cycle repetitions).

9.4. Preparation of the pre-amplified samples

- Thaw Target Solution (blue) and Control Solution (violet) in the refrigerator away from light or at ambient temperature, also away from light. Thaw PCR-grade water (white) at ambient temperature. Store reagents in cooling block.
- If necessary, briefly thaw pre-amplicons at ambient temperature or in the refrigerator, centrifuge, and store in the cooling block until further use.

Carry out the next steps under a clean PCR workbench.

- For each patient sample, prepare 60 µl of PCR-grade water (white) in a reaction tube.
- Add all three preparations of each sample to the PCR-grade water (3 × 20 µl pre-amplicon = 60 µl + 60 µl PCR-grade water).
- For negative control, prepare 20 µl of PCR-grade water in a reaction vial, and add the respective pre-amplicon.

9.5. Configuration of the qPCR plate

- Mix Target Solution (blue) and Control Solution (violet) for approx. 3 s on the vortex mixer, centrifuge, and store in the cooling block.
- Six wells are needed for each patient sample (three for the Target Solution, three for the Control Solution). Two wells for each negative control (Fig. 1).
- Pipette 15 µl of the Target Solution or Control Solution into the corresponding positions of the qPCR plate.
- Mix the diluted pre-amplicons on the vortex mixer for approx. 3 s before use, centrifuge, and store in the cooling block.
- Pipette 5 µl of the diluted pre-amplicons into the corresponding positions of the qPCR plate.
- Seal the qPCR plate with an optical sealing foil, and smooth it out with a film applicator so that it is free of bubbles.
- Centrifuge the PCR plate using a plate centrifuge (e.g. 2 × 30 s at 500 *g*).

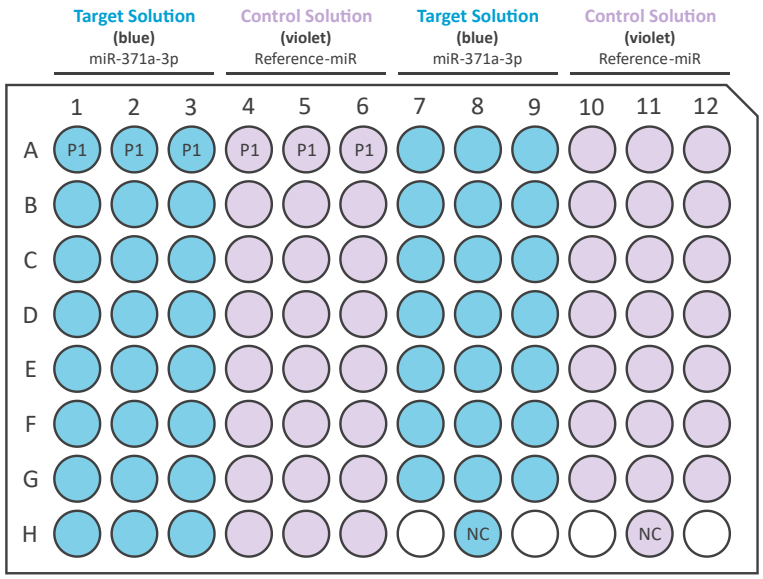


Fig. 1: Recommended plate assignment of the qPCR plate for the measurement of one sample (P1) and one negative control (NC).

9.6. Loading of the qPCR plate

Please refer to the manufacturer's information regarding the programming of your real-time PCR cycler.

- Create a qPCR programme in the qPCR Cycler software according to Table 9.
- The appliance-specific maximum heating and cooling rates can be maintained (Table 10).
- The following general settings must be made:
  Reaction volume: 20 µl
  Detection channel: FAM
- Open the loading flap of the qPCR instrument, and place the qPCR plate in the frame. Make sure that the panel fits exactly into the frame. Close the loading flap.
- Start the qPCR run and enter a clearly identifiable name.
- At the end of the run, remove the qPCR plate from the qPCR instrument, and discard it without removing the protective foil.

Special feature of LightCycler® 480II (Roche): In the plate loading schemes, the samples in the parallel measurements of miR-371a-3p and reference miR must be defined as triplicates to each other.

- Under the item “Sample Editor”, the three solutions for each patient sample and each miRNA must be marked as replicates. To do this, select three positions each, and click on the “Make Replicates” button
- Example: A1–A3 = one replicate, A4–A6 = one replicate (Fig. 1)

Table 9: qPCR temperature profile.

Number of cycles	**Step**	**Temperature [°C]**	**Duration [mm:ss]**	**Detection**
1	Polymerase activation	95	10:00	
40	Denaturation	95	00:15	
	Annealing/Elongation	60	01:00	Fluorescence measurement at the end of each cycle
1	Cooling	37	01:00	

Table 10: Device-specific heating and cooling rates of validated qPCR cyclers

qPCR cycler	**Maximum ramp rates [°C/s]**
LightCycler® 480 II	4,4 (heating), 2,2 (cooling)
QuantStudio™ 5	3,66
AriaDx	6,0

10. Result analysis

Note: Cp (= crossing point) and Ct (= cycle threshold) are identical and interchangeable. In these instructions for use, the term Ct is used.

10.1. M371-Test evaluation file and data import

The M371-Test evaluation file is an optional accessory software and is sent in electronic form **via email** after the kit has been purchased. The file labelled “M371-Test evaluation file” is available only in English. The latest version of the M371-Test evaluation file should always be used for reliable and safe evaluation. In order to ensure safe use, the M371-Test evaluation file contains locked areas, **which cannot and may not be changed**. Writeable cells (e.g. the name of the samples and the area for inserting data from the qPCR) are highlighted in light green. After inserting the data from the qPCR run into the M371-Test evaluation file, the RQ of miR-371a-3p is automatically calculated, and the test result is displayed.

10.2. Result analysis

Analysis settings:

Table 11: Device-specific analysis settings.

qPCR cycler	Threshold value	Base line
LightCycler® 480 II	Abs Quant/2nd Derivative Max	The start and end cycle must be selected so that initial signal noise is not taken into account and the baseline ends before significant fluorescence is detected.
QuantStudio™ 5	Auto threshold	
Aria Dx	Auto threshold	

The results analysis with the optional M371-Test evaluation file is described below. The procedure described here refers to the Roche Diagnostics LightCycler® 480 II qPCR instrument with 96-well heating block and software version 1.5.x. When using other validated qPCR cyclers, ensure that the median Ct values are transferred correctly to the input screen of the M371-Test evaluation file. The device-specific analysis settings of the validated qPCR cycler systems can be seen in Table 11.

- In the LightCycler® 480 software, select the previous experiment and click the “Analysis” tab.
- Select “Abs Quant/2nd Derivative Max” for all samples and click “OK”.
- Select “Median” instead of “Mean” in the drop-down menu on the lower right side and calculate by “Calculate” on the lower left side.
- The median Ct values of the miR-371a-3p and reference miR are automatically calculated for each sample and displayed in the “Replicate Statistics” results table at the bottom left.
- All results from the “Replicate Statistics” results table must be transferred to the M371-Test evaluation file. To do this, click in the field “Replicate Statistics”, select all data with Ctrl + A, and copy the data with Ctrl + C.
- Switch to the M371-Test evaluation file and follow the instructions there for data transfer.
- The results of the negative control for the miR-371a-3p and reference miR measurement must be entered manually into the evaluation file. By holding the cursor (mouse pointer) over the corresponding well position in the LightCycler® software, the measured Ct value is displayed.
- The evaluation file contains separate results columns for primary diagnostics and follow-up care. Because of different threshold values, the results in these columns may differ. **Make sure to read the result from the column corresponding to the respective scenario.**

10.2.1. Negative control

In each qPCR run, a negative control (NC, PCR-grade water) must be used for both the miR-371a-3p and the reference miR measurement in order to confirm the successful performance of the assay.

A qPCR run is **VALID** if the negative control for the miR-371a-3p and the negative control for the reference miR measurement are **NEGATIVE**. The negative control is negative if the Ct value for both miRNA samples measured is at least 10 cycles later than the highest value of the corresponding miRNA of a sample or is at a value of 35 or more.

A qPCR run is **INVALID** if the negative control is **POSITIVE**. The negative control for the miR-371a-3p and the reference miR measurement is positive if the Ct value for the respective specifically measured miRNA is less than 10 cycles later than the highest value of a sample.

If the negative controls are **POSITIVE**, the samples processed together with the controls **cannot** be evaluated. The M371-Test must be repeated for all samples in such a case.

The M371-Test evaluation file shows whether all controls passed (M371-Test evaluation file ➔ Controls: NC miR-371a and NC Reference miR).

10.2.2. Reference miR

The reference miR indicates whether a sufficient amount of miRNA from each sample was present in the respective preparation. The result of the miR-371a-3p qPCR is dependent on the result of the reference miR.

The normal range for the Ct value of the reference miR is between 12 and 22 with the LightCycler® 480 II instrument. In this case, sufficient miRNA is present, and the results are valid.

If the Ct value of the reference miR of a sample is **greater than 22**, this indicates low initial amounts after RNA extraction and may lead to an unambiguous diagnosis.

If the Ct value of the reference miR of a sample is **lower than 12**, haemolysis of the sample may have occurred, and a clear statement about the tumor status is not possible on the basis of this sample.

Patient samples with reference miR Ct values below 12 or above 22 should be taken again and processed with the M371-Test.

10.2.3. Assessment of the samples

The assessment of the test results depending on the clinical scenario is described in Chapter 2 “Technological basics of the test procedure”.

11. Troubleshooting guide

- If a negative control is not passed for a qPCR run, the entire run must be repeated (samples, including negative control for the miR-371a-3p and the reference miR measurement).
- Unwanted signals in the negative controls may be due to non-activated heating lid in cDNA synthesis or pre-amplification. Undesirable concentration shifts occur because of condensation on the lids of the reaction vessels. To avoid this, a heating lid temperature of 105°C should be programmed.
- If, for a sample, the Ct value of the reference miR is above 22, the sample should be taken again and processed with the M371 test because the amount of starting material was insufficient.
- If the Ct value of the reference miR is below 12 for a sample, the sample should be re-collected and processed with the M371-Test because the original sample was probably haemolytic.
- If the RQ for a sample from **primary diagnostics** is between 5 and 10 (indeterminate range), please take another blood sample from the patient after a few weeks and repeat the measurement.
- LightCycler® software: If the “Replicate Stats” table is missing, check whether the replicates of a patient sample are assigned to each other as replicates.

12. Limits of the procedure

- The test is suitable only for *in vitro* diagnostics.
- The test is exclusively designed for the detection of Type II testicular germ cell tumors (Germ Cell Neoplasia *in situ* derived GCTs).
- The test has not shown any prognostic function (prediction of recurrences after intervention) but can be used for the follow-up monitoring of testicular tumor patients.
- Only the qPCR cyclers mentioned in Chapter 5.1. General laboratory equipment were validated.
- This product was developed for the analysis of serum. Only the S-Monovette® serum gel blood collection tubes (7.5 and 9 ml Z-Gel) from Sarstedt AG & Co. KG were validated.
- Other types of patient samples and other blood collection tubes have not been validated.
- The instructions for sampling and sample processing in Chapter "8. Sample collection and sample processing" must be observed.
- This product may be used only by persons with experience in performing PCR tests.
- The test result cannot be used as the sole primary diagnosis of a testicular germ cell tumor or to detect recurrence. Any positive M371-test should be confirmed by an appropriate clinical diagnostic procedure.
- The result of the M371-Test must be assessed in the context of other clinical parameters.
- Pure teratomas show virtually no increased expression of the tumor marker miR-371a-3p. This is why this tumor entity is not detectable (Dieckmann et al., 2017; 2019).
- The reference miR is expressed at higher levels in the brain tissue of Alzheimer's patients (Song et al., 2019). It is currently not known whether this also applies to the concentration of the reference miR in the serum of these patients. In this case, false-negative test results may occur.
- Positive test results have been observed in pregnant women. However, they do not belong to the target group of patients to be analysed with the M371-Test (Gu et al., 2013).
- Increased haemolysis leads to an increased release of the reference miR detected in the test. This results in a considerable reduction of the Ct values of the reference miR and can thus lead to falsified RQ values and, in the worst case, to a false-negative test result (Myklebust et al., 2019).
- It cannot be ruled out that miR-371a-3p is expressed at an increased level in COVID-19 patients (Goebel et al., 2022).

13. Specific performance data

13.1. Analytical performance

13.1.1. Analytical sensitivity

The smallest measurable difference in RQ values and Ct values was measured using three dilution steps of mimic miRNA samples consisting of miR-371a-3p and reference miR. Each dilution was measured in 10 replicates with one kit batch. This resulted in the smallest measurable difference being 0.52 pmol/l.

13.1.2. Analytical specificity

Three different patient simulating samples (high, medium, no miR-371a-3p expression) with high, low, and no contamination (DNA, protein contamination) were measured. The same M371-Test kit batch was used for all measurements. The results were examined through a regression analysis.

The Ct value of miR-371a-3p increased in highly expressing samples because of contamination. This can lead to a lower RQ ($p = 0.005$, $R^2 = 0.698$).

In moderate expressing samples, DNA/protein contamination resulted in significantly higher Ct values of miR-371a-3p and reference miR as well as RQ values ($p = 0.001$, $R^2 = 0.798$; $p = 0.004$, $R^2 = 0.711$; $p = 0.001$, $R^2 = 0.812$).

In view of the results, special attention should be paid to correct miRNA extraction in accordance with the manufacturer's protocol in order to avoid possible contamination of a patient sample.

13.1.3. Limit of detection (LoD) and limit of quantification (LoQ)

The limit of detection (LoD) and limit of quantification (LoQ) of the M371-Test was determined in a dilution series of the miR-371a-3p with six dilution levels as well as the reference miR at a constant concentration with six replicates each. All measurements were carried out with a single M371-Test kit batch.

The limit of detection (LoD) was defined in advance in such a way that at least 5/6 dilutions must be detectable. This was the case in the experiment up to a concentration of 7.575 fM. The coefficient of variation was 77.33%.

The limit of quantification (LoQ) was defined in advance in such a way that the coefficient of variation should not exceed 50%. This was the case up to a concentration of 30.3 fM. For this concentration, the coefficient of variation is 44.07%. The mean RQ at the LoD is 1.05; the mean RQ at the LoQ is 8.71. This means that the LoQ is just above the cut-off value of RQ = 5. Because values below 8.71 cannot be quantified accurately, the cut-off value for the primary diagnosis was expanded to a cut-off range that includes RQ values from 5 to 10. Values within this range cannot be measured accurately and are considered INDETERMINATE.

13.1.4. Linearity

For the measurement of linearity, a mimic-miRNA sample at a concentration of 500 pM was diluted 1:10 six times. Each dilution was measured three times independently by the same operator using a single M371-Test kit batch on different days. This resulted in an average PCR efficiency of 90%; the correlation coefficient (R^2) was 0.993–0.997. When looking at miR-371a-3p Ct values, concentrations from 5 fM to 500 fM were in the linear range. At a concentration of 0.5 fM, miR-371a-3p was not detectable.

13.2. Precision

13.2.1. Repeatability

The reproducibility of the test results was determined by repeatedly testing samples with four different concentrations (high, medium, low, and no miR-371a-3p expression). Each sample was processed in 30 replicates with a single batch of the M371-Test kit by one operator. The coefficient of variation for samples with high and medium expression is approx. 14%. For low expressing samples, the coefficient of variation is up to 85%; the limit of quantification must therefore be considered in the evaluation. Samples representing tumor-free patients can have a coefficient of variation of up to 127%. This was observed at a concentration of 5 fM. Because this concentration is below the detection limit (7.575 fM), a higher coefficient of variation is unproblematic.

13.2.2. Reproducibility

The following parameters were examined for the reproducibility:

- Different operators
- Different consumables (qPCR plates)
- Different laboratories (different PCR cycler and qPCR cycler instruments (LightCycler® 480II)

Four different concentrations of samples (high, medium, low, and no miR-371a-3p expression) were measured per operator in two replicates. Four concentrations (high, medium, low and no miR-371a-3p expression) were measured per plate type with four replicates each. Four concentrations (high, medium, low, and no miR-371a-3p expression) were measured per laboratory, each with four replicates.
Operators and consumables such as qPCR plates had no significant influence on the RQ of the samples tested ($p = 0.09–0.33$, Kruskal Wallis and $p = 0.25–0.81$, Mann-Whitney U, respectively). When comparing two laboratories, there was a significant difference in the higher range of RQ ($p = 0.014$, Mann-Whitney U in the RQ range of 200–2,000). However, this did not affect the range of the clinical decision limit (RQ = 10) and was in the range of 21–22% for the coefficient of variation.

13.3. Clinical performance

The clinical performance of the M371-Test was demonstrated, among others, in a multi-centre study at 37 clinics from Germany, Austria, Switzerland, Hungary, and Italy (Dieckmann et al., 2019). For the study, serum samples from 616 patients with germ cell tumors and from 258 control patients were measured with the M371-Test. To determine the clinical performance for primary diagnostics, samples from 522 tumor patients (of which 323 seminomas and 199 non-seminomas) were compared with samples from 258 control patients.

In the primary diagnosis of germ cell tumors, the M371-Test showed a sensitivity of 91.8% and a specificity of 96.1%. The AUC (area under the ROC curve) was 0.970, and the positive predictive value was 97.2% (Table 12).

Table 12: Clinical performance characteristics of the M371-Test (from Dieckmann et al. 2019).

Group	AUC	Sensitivity	Specificity	PPV	NPV	LR+	LR−
GCT (n = 522) vs controls (n = 258)	0,970 (0,958–0,981)	91.8 (89.1–94.0)	96.1 (93.0–98.1)	97.2 (92.9–99.2)	82.7 (74.0–89.45)	23,675 (12.89–43.49)	0,086 (0.06–0.11)
Seminomas (n = 323) vs controls (n = 258)	0,964 (0,949–0,979)	89.8 (85.9–92.8)	96.1 (93.0–98.1)	-	-	-	-
Non-seminomas (n = 199) vs controls (n = 258)	0,978 (0,962–0,994)	95.0 (91.0–97.6)	96.1 (93.0–98.1)	-	-	-	-
CS I (n = 371) vs controls (n = 258)	0,958 (0,942–0,974)	88.9 (85.3–92.0)	96.1 (93.0–98.1)	-	-	-	-
CS II/III (n = 151) vs controls (n = 258)	0,998 (0,995–1.0)	98.7 (95.3–99.8)	96.1 (93.0–98.1)	-	-	-	-
Recurrences (n = 46) vs controls (n = 258)	0,921 (0,862–0,981)	82.6 (68.6–92.2)	96.1 (93.0–98.1)	-	-	-	-

*The clinical performance measures for the PPV and NPV are based on n = 155 GCT and n = 90 controls. AUC: area under the curve, CS: clinical stage, GCT: germ cell tumor, LR+: positive likelihood-ratio, LR−: negative likelihood-ratio, PPV: positive predictive value, NPV: negative predictive value. Values in brackets = 95% confidence interval.

Recurrences of GCT patients were correctly determined in 10 out of 10 and 4 out of 4 recurrences, respectively, using increased miR-371a-3p expression (Dieckmann et al., 2017; van Agthoven et al., 2017). Another group showed an increase in miR-371a-3p expression during relapse in samples from 10 TGCT patients (Terbuch et al., 2018).

Dieckmann et al. demonstrated a sensitivity of 83% in n = 46 TGCT recurrences with normalisation of miR-371a-3p serum levels after successful treatment of relapses (Dieckmann et al., 2019). An increase in miR-371a-3p at relapse was also noted by Rosas Plaza et al. (Rosas Plaza et al., 2019).

In a series of n = 151 clinical stage 1 TGCT patients, Lobo and colleagues found n = 34 cases of relapse. Of these, they were able to detect n = 32 (94%) with the miR-371a-3p measurement whilst the classic gold standard (AFP and βHCG) was elevated in only 38% of the cases (Lobo et al., 2020).

The reliability with which miR-371a-3p expression detects recurrences was further confirmed by Fankhauser et al. In a study of 30 patients, increased miR-371a-3p expression was found in 10/10 patients with recurrence, whereas miR-371a-3p was increased in only one patient without recurrence (Fankhauser et al., 2022). This increase normalised by the next measurement, thereby indicating that even after increased expression, the increase in miR-371a-3p should be monitored. Recurrences were

measured a median of two months earlier than with the conventional methods and in one patient even more than five months earlier (Fankhauser et al., 2022).

The currently most comprehensive study on the performance of the M371-Test in the follow-up of patients with clinical stage I testicular germ cell tumors included 258 patients and observed them over a median period of 18 months. The test was positive in all 39 patients who developed a recurrence during this period. Recurrences were detected with a sensitivity of 100% and a specificity of 96.3% (Belge et al., 2024). In this follow-up study, a relative expression of RQ = 15 was used as a cut-off for detecting recurrences; this differs from the cut-off used in the primary diagnosis (RQ = 5, Dieckmann et al., 2019) (see also "2. Technological basics of the test procedure"). Although the classic serum markers bHCG and AFP were able to detect recurrences with a specificity of 91.8%, they achieved a sensitivity of only 45.2%. Further details on the sensitivity and specificity as well as the positive and negative predictive values of the M371-Test compared with the classic serum markers in the follow-up of patients with clinical stage I germ cell tumors are summarised in Table 13.

Table 13. Clinical performance characteristics of the M371-Test in the follow-up of CS I GCT patients in comparison with the classic serum markers (from Belge et al., 2024).

	Clinically confirmed recurrences				Recurrence-free cases				PPV (%)	NPV (%)
Markers	n	TP	FN	Sensitivity (%)	n	TN	FP	Specificity (%)		
M371, all GCT	39	39	0	100.0 (100.0–100.0)	219	211	8	96.3 (93.9–98.8)	83.0 (72.2–93.7)	100.0 (100.0–100.0)
M371 S	17	17	0	100.0 (100.0–100.0)	172	166	6	96.5 (93.8–99.3)	73.9 (56.9–91.8)	100.0 (100.0–100.0)
M371, NS	22	22	0	100.0 (100.0–100.0)	47	45	2	95.7 (92.9–100.0)	91.7 (80.7–100.0)	100.0 (100.0–100.0)
bHCG	31	11	20	35.5 (19.2–54.6)	196	192	4	98.0 (94.9–99.4)	73.3 (51.0–95.7)	90.6 (86.6–94.5)
AFP	32	8	24	25.0 (11.5–43.4)	196	184	12	93.9 (89.5–96.8)	40.0 (18.5–61.5)	88.5 (84.1–92.8)
bHCG/AFP	31	14	17	45.2 (27.3–64.0)	196	180	16	91.8 (87.1–95.3)	46.7 (28.8–64.5)	91.4 (87.4–95.3)

GCT: Germ cell tumor, S: Seminoma, NS: Non-seminoma, bHCG/AFP: Cases with at least one positive serum marker bHCG and/or AFP, n: recorded number of cases, TP: true positive, FN: false negative, TN: true negative, FP: false positive, NPV: negative predictive value, PPV: positive predictive value. Values in brackets = 95% confidence interval.

13.4. Interference

13.4.1. Haemolysis

Increased haemolysis leads to an increased release of the reference miR detected in the test. This results in a considerable reduction of the Ct values of the reference miR and can thus lead to falsified RQ values and, in the worst case, to a false-negative test result. Twenty sera samples from patients were analysed for haemolysis by staining and by photometric measurement (414 nm). Each sample was measured once with the M371-Test. The degree of haemolysis had a significant influence on the measurement of the reference miR ($p = 0.002$). A higher degree of haemolysis results in a lower Ct value of the reference miR ($R^2 = 0.437–0.743$).

13.4.2. Other medical conditions

Increased concentrations of the reference miR in brain tissue have been observed in patients with Alzheimer's disease (Song et al., 2019). It is currently not known whether there are also elevated concentrations of the reference miR in the serum of these patients; these could falsify the test result. Positive test results were observed in pregnant women, although they do not belong to the target group of patients to be analysed. Recent studies suggest that miR-371a-3p could be increased in patients with COVID-19 disease (Goebel et al., 2022). If these studies are confirmed, it is recommended that the COVID-19 status of the patients be investigated in parallel in cases of suspicion.

13.4.3. Cross-reactivity

The following substances were tested for interference with the M371-Test: DNA contamination, proteins, EDTA, citrate, heparin, and similar miR sequences (miR-372-3p).
CLSI Interference Testing in Clinical Chemistry 3rd ed. was used for interference testing. Initially, a serum sample for each interferent was divided into two groups, one of which was enriched with a concentration of interferent three times higher than would normally be expected. The other group was not enriched with interferents and served as a control. Each group was measured in seven replicates. If the difference in the result exceeded a predetermined level (50%) of the test group compared with the control group, a dose-response experiment was conducted.
Contamination with DNA, protein, and heparin had an influence on the RQ and the result of the test even at relatively low levels of contamination.

Although the recommended miRNA isolation and similar methods remove DNA and protein from serum samples, failure to follow the manufacturer's protocol may result in contamination of patient samples with DNA or protein. This contamination can lead to falsified results. mir|detect GmbH therefore strongly recommends strictly following the manufacturer's protocols.

Because even small amounts of heparin can influence the results of patient samples, mir|detect GmbH recommends using Sarstedt AG & Co. KG S-Monovette® serum gel for blood collection or serum collection

It cannot be ruled out that further possible interference will be detected.

13.5. Summary of Safety and Performance

The current summary report for safety and performance ("Summary of Safety and Performance") can be viewed via EUDAMED (https://ec.europa.eu/tools/eudamed/#/screen/home) or requested using the contact form at www.mirdetect.de/Service.

14. Meaning of symbols

The use of symbols is based on DIN EN ISO 15223-1:2021 (Medical devices – Symbols to be used with information to be supplied by the manufacturer – Part 1: General requirements (ISO 15223-1:2016, corrected version 2017-03); German version EN ISO 15223-1:2016).

The symbols and their meanings are shown below (Table 14).

Table 14: Representation of symbols and their meanings.

CE XXXX	CE marking + code for notified body (XXXX)
IVD	*In vitro* diagnostic medical device
	Follow the instructions for use
REF	Item number
LOT	Batch number
	Manufacturer
	Distributor
	Sufficient for <n> tests
	Protect from sunlight
	Temperature limiter
	Can be used until
	Do not use if the packaging is damaged

15. Changes to previous instructions for use

15.1. Changes to version 10

- Update of the distributor company name
- Addition of a table for the interpretation of the M371-Test results depending on the clinical scenario in Chapter 2 “Technological basics of the test procedure”
- Explanation of the RQ calculation in Chapter 2 “Technological basics of the test procedure”
- Reference to the documents available for download on the mir|detect website in Chapter 3.3 “Accessories”
- Addition of Chapter 3.4 “Hazardous substances and animal components in the M371-Test kit”
- Correction of the transport temperature in Chapter 4
- New illustration for qPCR plate loading sequence in Chapter 9.5
- Removal of the “non-sterile” symbol from the table in Chapter 14
- Update of Chapter 10.2 “Result analysis” (LightCycler instrument)
- Update of Chapter 12 “Limits of the procedure”
- Update of Chapter 13 “Specific performance data”

15.2. Changes to version 11

- Correction of formulation and spelling errors
- Addition of the Notified Body code next to the CE symbol on the cover sheet
- Reference to IVDR certification of the M371-Test in Chapter 1
- Listing of the reactive components of the M371-Test in chapter 3.2
- Recording of the real-time PCR cycler QuantStudio 5 (Thermo Fisher Scientific) with 96-well heating block and “Design and Analysis” software, Version 1.4.x and Aria Dx (Agilent) with software version 2.0 in Chapter 5.1
- Removal of the optional positive sample from Chapters 3 and 9, the associated tables, and Chapter 18.1 for the master mix preparation calculation
- Replacement of the “Accessories” chapter with Chapters “3.3 Information and documents for the M371-Test” and “3.4 Optional accessories”
- Note on the optional M371-Test evaluation file, which is available only as the English version “M371-Test evaluation file” in Chapters 3.4 and 10.1
- Presentation of the important notes in Section 9.1 in sub-sections for better clarity and the addition of two new notes on the use of cooling block /cooling racks and activating the heated lid on PCR cyclers.
- Change of the designation “Primer hybridisation” to “Annealing” in Tables 6 and 8
- Note on activating the heated lid in Chapters 9.2, 9.3, and 11
- New note on mixing and centrifugation of pre-amplicons in Chapter 9.5
- Addition of a table for the heating and cooling rates of the validated cycler systems and simplification of the table for the qPCR cycler temperature profile in chapter 9.6
- Reference to the two separate results columns in the M371-Test evaluation file and addition of a table for the device-specific analysis settings of validated cycler systems in chapter 10.2
- More detailed description of the increased expression of the reference miR in Alzheimer’s patients in Chapter 12 and 13.4.2
- Correction of the analytical sensitivity to 0.52 pmol/l in Chapter 13.1.1
- Additional list of changes to Version 10 in Chapter 15.1
- Update of the mail address of the distributor in Chapter 17.3

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17. Information for the purchaser

17.1. Manufacturer

mir|detect GmbH
Fischkai 1
27572 Bremerhaven, Germany

For further information and assistance, please visit our website, send us an email, or call us:

Website: https://www.mirdetect.de
Email: info@mirdetect.de
Telephone: +49 (0) 421 / 40 89 37 11-0

17.2. Trademarks

All trademarks, brands, and names mentioned in this document are the property of the respective companies.

17.3. Distributor

Gold Standard Diagnostics Frankfurt GmbH
Waldstraße 23 A6
63128 Dietzenbach

Email: Info.Frankfurt@eu.goldstandarddiagnostics.com
Phone: +49 (0) 6074 23698-0

18. Appendix

18.1. Templates for the preparation of the cDNA synthesis master mix (MM)

Table 15: Pipetting scheme for the preparation of a cDNA synthesis master mix for 2, 3, 4, and 5 samples (including 10% excess volume), Rxn = reactions.

Master mix (MM) / Reagent	MM (2 samples)	MM (3 samples)	MM (4 samples)	MM (5 samples)
	3 Rxn (including NC and excess) (µl)	4 Rxn (including NC and excess) (µl)	5 Rxn (including NC and excess) (µl)	6 Rxn (including NC and excess) (µl)
cDNA solution (black)	25.77	34.36	42.96	51.55
Reverse Transcriptase (yellow)	3.3	4.4	5.5	6.6
RNase Inhibitor (transparent)	0.63	0.84	1.05	1.25
Total volume	**29.7**	**39.6**	**49.5**	**59.4**