Instructions for use

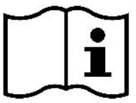
M371-Test

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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1. Name and intended use of the product

The laboratory diagnostic medical device M371-Test is an 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 serum from the cubital vein by qPCR.

The M371-Test is a non-automated test with a qualitative interpretation of results that detects the presence of testicular germ cell tumors (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, Germ Cell Neoplasia in situ derived TGCT). The test result cannot be used for the sole primary diagnosis of a testicular germ cell tumor. 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 M371-Test evaluation file is provided for the evaluation of the samples.

The detection method is based on the fluorescence-induced detection of the microRNA miR-371a-3p by quantitative PCR. In order to measure this tumor marker, RNA, including microRNA, 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 microRNA 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 abundance of miR-371a-3p is calculated according to the ΔΔCt method (Livak & Schmittgen, 2001) by the reference miR and a fixed value (calibrator).

The M371-Test can lead to three different results:

RQ < 5 = negative, low tumor probability
RQ 5–10 = undetermined; repetition after a few weeks recommended
RQ > 10 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 performed with a negative control (NC) and, if desired, a positive sample (PS). 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 and, if desired, a positive sample (see Table 1).

CAUTION: Negative and positive samples are measured only **once** each in the pre-amplification and qPCR.

Table 1: 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, one negative control and, if desired, one positive sample. The user must measure all samples in **one** run (see Table 2).

Table 2: 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 µl

3.2. Reactive components of the M371-Test

A reverse transcriptase is used for reverse transcription during cDNA synthesis. The M371-Test component “PreAmp Solution” contains TaqMan™ microRNA Assay. This reagent contains miRNA-specific primers and TaqMan™ probes. The two components “Target Solution” and “Control Solution” contain TaqMan™ microRNA assay and DNA polymerase.

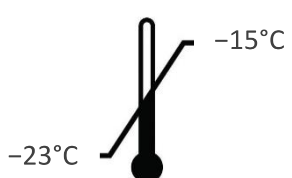
3.3. Accessories

The M371-Test evaluation file (spreadsheet software with stored formulas) for the evaluation of samples and the instructions for use are transmitted in electronic form (via email).

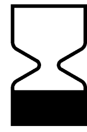
The instructions for use, safety data sheets and video tutorials are also available in the service area at www.mirdetect.de/Service available.

4. Transport, storage, and stability

The M371-Test is shipped < 5°C via express shipping. **In the event of transport damage, please contact both the transport company and Novatec Immundiagnostica GmbH, part of Gold Standard Diagnostics Frankfurt, immediately**. 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.

- Spreadsheet 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 M371-Test evaluation file was validated using Microsoft Excel 2019, 2003 as well as Apache OpenOffice 4.1.5.

** The M371-Test was validated with Roche Diagnostics LightCycler® 480 II qPCR instrument with 96-well heating block and software version 1.5.x

5.2. General consumables and reagents

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

- Blood tubes*
- Cryotube, self-standing
- RNA/miRNA extraction kit**
- 1.5 ml reaction tubes with conical bottom and safety lid (PS)
- 0.2 ml PCR reaction tubes (8-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).
** necessary for the extraction of miRNA. Recommended assay is QIAGEN GmbH miRNeasy Serum/Plasma Kit and Promega Corporation Maxwell RSC with miRNA Plasma and Serum Kit.

5.4. 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

Compliance with Good Laboratory Practices (GLP) is required in order to prevent the 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. Pipetting of the qPCR plate should not be done under direct light irradiation.

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 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 or similar tubes without further additives should be used for blood collection according to the manufacturer’s instructions.
- 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 on edge for 30 minutes at ambient temperature (15–25°C).

- Centrifuge the blood tube at 2500 × g for 10 minutes.
- 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 Coats (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 microRNA or RNA.

8.4. miRNA extraction

Materials for the extraction of RNA or miRNA from patient serum are not part of 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.

Caution: 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.
- **Caution:** 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, contact Novatec Immundiagnostica GmbH, part of Gold Standard Diagnostics Frankfurt (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 tests.

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, also analyzed as a single replicate in the final miR-371a and reference miR measurement.

Note: Mix ALL solutions of the kit – excluding Reverse Transcriptase (yellow) and RNase Inhibitor (clear) – on a Vortex Mixer for approx. 3 s at approx. 2,800 rpm before use in order to ensure a homogeneous solution.

Note: Centrifuge ALL solutions of the kit – including Reverse Transcriptase (yellow) and RNase Inhibitor (clear) – for approx. 3 s at 2.000 × g in order to remove drops on the lid before use.

Note: 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.

9.2. Performance of the cDNA synthesis

- Thaw cDNA Solution (black) and PCR grade water (white) at ambient temperature.
- 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 the cDNA Solution, the Reverse Transcriptase, and the RNase Inhibitor together in a suitable reaction vessel according to the number of samples required. Note the ratio from Table 3.
- 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 (see Table 4).
- Add 6 µl sample or control each.

Table 3: Pipetting scheme for the preparation of a cDNA synthesis master mix (MM)
Rxn = reactions

Mastermix (MM) / Reagent	Single sample	MM (2 samples)
	1 Rxn [µl]	**4 Rxn (including NC and optional PS + 10% excess) [µl]**
cDNA Solution (black)	7.81	34.36
Reverse Transcriptase (yellow)	1.00	4.4
RNase Inhibitor (transparent)	0.19	0.84
Total volume	**9.00**	**39.6**

Table 4: Distribution of the cDNA synthesis Mastermix (MM) and samples into the PCR reaction tubes (8-tube strips). Illustration of the performance for two samples, a negative control (NC), and a positive sample (PS).

PCR reaction vessels
MM + Sample 1
MM + Sample 2

MM + NC
MM + PS*

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*optional

- 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 5.
- Finished cDNA can be stored overnight in the refrigerator (4°C). Freeze at −20°C for longer storage.

Table 5: Parameters of the cDNA synthesis program for a standard PCR instrument.

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

9.3. Performance of the pre-amplification

- Thaw PreAmp Solution (green) at ambient temperature. 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 (see Table 6).
- For the negative control and positive sample (optional), 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 7.
- Finished pre-amplicons can be stored overnight in the refrigerator (4°C). Freeze at −20°C for longer storage.

Table 6: Illustration of the performance of a pre-amplification for two samples, a negative control (NC), and a positive sample (PS).

PCR reaction vessels
Sample 1
Sample 1
Sample 1
Sample 2
Sample 2
Sample 2
PS*
NC

*optional

Table 7: 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	Primer hybridisation + elongation
1	≤ 10	∞	Cooling down

9.4. Preparation of the pre-amplified samples

- Thaw Target Solution (blue) and Control Solution (violet) at ambient temperature away from light. Thaw PCR-grade water (white) at ambient temperature. Store reagents in cooling block.
- If necessary, thaw pre-amplicons at ambient temperature, 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 fresh reaction tube.
- Add all three mixtures of each sample to the PCR-grade water. This corresponds to a 1:1 dilution (3 × 20 µl pre-amplicon = 60 µl + 60 µl PCR-grade water).
- For negative and positive samples, prepare 20 µl of PCR-grade water in a fresh reaction tube and add the pre-amplicons.

9.5. Loading 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 and positive sample (see Table 8).
- Pipette 15 µl of the Target Solution or Control Solution into the corresponding positions of the qPCR plate.
- Pipette 5 µl of the pooled and diluted pre-amplicons into the corresponding positions of the qPCR plate.
- Seal the qPCR plate with an optical cover film, and smooth it out with a film applicator so that it is free of bubbles.
- Centrifuge the PCR plate for 1 min at 500 × g with a plate centrifuge.

Table 8: Recommended plate assignment of the qPCR plate for the measurement of two samples (P1, P2), one negative control (NC), and a positive sample (PS).

	miR-371a			**Reference miR**			**miR-371a**			**Reference miR**		
	Target Solution (blue)			**Control Solution (violet)**			**Target Solution (blue)**			**Control Solution (violet)**		
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	P1	P1	P1	P1	P1	P1	P2	P2	P2	P2	P2	P2
B	---	---	---	---	---	---	---	---	---	---	---	---
C	---	---	---	---	---	---	---	---	---	---	---	---
D	---	---	---	---	---	---	---	---	---	---	---	---
E	---	---	---	---	---	---	---	---	---	---	---	---
F	---	---	---	---	---	---	---	---	---	---	---	---
G	PS*	---	---	PS*	---	---	---	---	---	---	---	---
H	---	NC	---	---	NC	---	---	---	---	---	---	---

*optional

9.6. Loading of the qPCR plate

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

- Create a qPCR program in the qPCR Cycler software according to Table 9.
- 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
- Ex: A1–A3 = one replicate, A4–A6 = one replicate (see Table 8)
- 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 lid of the sample chamber.
- Start the qPCR run by clicking on “Start Run”, and enter the name to be uniquely identified.
- At the end of the run, remove the qPCR plate from the qPCR instrument, and discard it without removing the protective foil.

Table 9: qPCR program for the LightCycler® 480 II instrument.

Program parameters	Enzyme activation	PCR		Cooling down
96-well block	Heating rate 4.4°C/s and cooling rate 2.2°C/s			
Analysis mode	None	Quantification mode		None
Cycles	1	40		1
Step	1	1	2	1
Target temperature [°C]	95	95	60	37
Duration [hh:mm:ss]	00:10:00	00:00:15	00:01:00	00:01:00
Heating rate [°C/s]	4.4	4.4	2.2	2.2
Detection mode	none	none	one-off	none
Fluorophore	none	none	FAM*	none

*Filter set: excitation 465 nm and emission 510 nm

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 integral part of the M371-Test as software and is transmitted in electronic form (via email) when the kit is purchased. For safe use of the M371-Test evaluation file, the locked areas of the file may not be changed. A current version of the spreadsheet software should be used.

In the M371-Test evaluation file, writable cells are highlighted in light green (e.g. designation of samples and insertion of data from qPCR); all other cells are locked and may not be changed.

After inserting the data from the qPCR run into the evaluation file, the RQ of miR-371a is automatically calculated, and the test result is displayed.

10.2. Result analysis (LightCycler® 480 II instrument)

For this step, the M371-Test evaluation file provided by mir|detect is required. 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.

- In the LightCycler® 480 Basic 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 and reference miR are automatically calculated for each sample and displayed in the “Replicate Statistics” results table at the bottom left.
- Transfer all results from the “Replicate Statistics” results table 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.
- Click on the “Samples” field, and insert the data with Ctrl + V (Insert).
- The results of the negative control for the miR-371a 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.

10.2.1 Negative control

In each qPCR run, a negative control (NC, PCR grade water) must be used for both the miR-371a 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 and the negative control for the reference miR measurement are **NEGATIVE**. The negative control is negative if the Ct value for both microRNA 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 and the reference miR measurement is positive if the Ct value for the respective specifically measured microRNA 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 run.

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

10.2.2 Reference miR

The reference miR indicates whether a sufficient amount of microRNA from each sample was present in the respective preparation. The result of the miR-371a 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

A sample is POSITIVE if the RQ is greater than 10.

A sample is NEGATIVE if the RQ is less than 5.

A clear statement for patients 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.

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 and the reference miR measurement).
- 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 is between 5 and 10, please draw fresh blood from the patient after a few weeks, and then analyze the serum again with the M371-Test.
- LightCycler® software: If the table “Replicate Stats” 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 GCNis).
- Only the qPCR cyclers mentioned in Chapter 5.1. General laboratory equipment were validated. Other qPCR cyclers must be validated by the user before use.
- 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.
- This product should only be used by persons with experience and training in PCR testing.
- The test result cannot be used for the sole primary diagnosis of a testicular germ cell tumor. 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.
- The reference miR is differentially expressed in AD patients; this can lead to false results (Song et al., 2019).
- Positive test results have been observed in pregnant women. However, they do not belong to the target group of patients to be analyzed with the M371-Test (Gu et al., 2013).

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 dilutions of mimic miRNA samples consisting of miR-371a and reference miR. Each dilution was measured in 10 replicates with one kit batch. This resulted in the smallest measurable difference being 2.56–3.08 pmol/l at a concentration of 61.5 pmol/l.

13.1.2 Analytical specificity

Three different patient-simulating samples (high, medium, no miR-371a expression) with high or low contamination (DNA, protein contamination) were measured pure. 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 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 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 and the reference miR with six dilution levels with six replicates each. All measurements were carried out with a single M371-Test kit batch.

The limit of detection (LoD) was defined in the study protocol such that at least 5/6 dilutions should 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 miR-371a. 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 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-Ct values, concentrations from 5 fM to 500 fM were in the linear range. At a concentration of 0.5 fM, miR-371a 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 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.

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 expression) were measured per operator in two replicates. Four concentrations (high, medium, low and no miR-371a expression) were measured per plate type with four replicates each. Four concentrations (high, medium, low, and no miR-371a 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 were compared with samples from 258 control patients. Clinical performance was evaluated using both the empirical data collected in the study and a kernel density estimate. This mathematical procedure was used to model the clinical performance characteristics of the M371-Test for an unlimited sample size. Because density estimation generally gives more conservative results, only these performance characteristics are given in Table 10.

Table 10: Clinical performance characteristics of the M371-Test from the kernel density estimate.

Group	AUC	Sensitivity	Specificity	PPV*	NPV*	LR+	LR−
GCT (n = 522) vs controls (n = 258)	0.966 (0.953–0.976)	90.1 (86.9–91.7)	94.0 (91.4–96.8)	97.2 (92.9–99.2)	82.7 (74.0–89.4)	23.675 (12.89–43.49)	0.086 (0.06–0.11)
Seminomas (n = 323) vs controls (n = 258)	0.959 (0.94–0.972)	87.1 (82.6–89.3)	94.0 (91.4–96.6)	-	-	-	-
Non-seminomas (n = 199) vs controls (n =258)	0.976 (0.958–0.989)	94.1 (90.2–96.4)	94.0 (91.3–96.8)	-	-	-	-
CS I (n = 371) vs controls (n = 258)	0.953 (0.934–0.967)	86.7 (82.3–88.8)	94.0 (91.3–96.8)	-	-	-	-
CS II/III (n = 151) vs controls (n = 258)	0.996 (0.992–0.999)	98.4 (96.2–99.5)	94.0 (91.4–96.9)	-	-	-	-

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, NPV: Negative predictive value, PPV: Positive 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 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 X. et al. (Rosas Plaza X. 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 expression detects recurrences was further confirmed by Fankhauser et al. In a study of 30 patients, increased miR-371a expression was found in 10/10 patients with recurrence, whereas miR-371a 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 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).

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 analyzed 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

Alzheimer's disease in patients can also lead to false-negative results. For this cluster of symptoms, the reference miR is also elevated and falsifies the test result. Positive test results were also observed in pregnant women, although they do not belong to the target group of patients to be analyzed. Recent studies suggest that miR-371a-3p is increased in patients with COVID-19 disease (Goebel et al., 2022). If these studies are confirmed, it is recommended that patients' COVID-19 status 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, citrates, 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 or a similar serum gel tube without additives.

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

13.5. Brief for safety and performance

The current summary report for safety and performance can be viewed via EUDAMED or requested via the contact form on www.mirdetect.de/Service.

14. Meaning of symbols

The use of symbols is based on DIN EN ISO 15223-1 (2016) (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 (see Table 11).

Table 11: Representation of symbols and their meanings.

CE	CE marking
IVD	*In vitro* diagnostic medical device
	Follow the instructions for use
REF	Item number
LOT	Production lot number, batch
	Manufacturer
	Distributor
	Sufficient for <n> tests
	Protect from sunlight
	Temperature limiter
	Can be used until
NON STERILE	Non-sterile
	Do not use if the packaging is damaged

15. Changes to the previous instructions for use (Version 09)

- Correction of the concentrations for LoD and LoQ in chapter 13.1.3. Limi of detection and limit of quantification (LoD, LoQ)

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17. Information for the purchaser

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17.2. Trademarks

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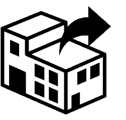
17.3. Distributor



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18. Appendix

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

Table 12: Pipetting scheme for the preparation of a cDNA synthesis master mix for 2, 3, 4, and 5 samples (including 10% excess volume), Rxn = reactions.

Mastermix (MM) / Reagent	MM (2 samples)	MM (3 samples)	MM (4 samples)	MM (5 samples)
	4 Rxn (including NC and optional PS + excess) [µl]	5 Rxn (including NC and optional PS + excess) [µl]	6 Rxn (including NC and optional PS + excess) [µl]	7 Rxn (including NC and optional PS + excess) [µl]
cDNA Solution (black)	34.36	42.96	51.55	60.14
Reverse Transcriptase (yellow)	4.4	5.5	6.6	7.7
RNase Inhibitor (transparent)	0.84	1.05	1.25	1.46
Total volume	**39.6**	**49.5**	**59.4**	**69.3**