Mantle cell lymphomas are a sub-type of non-Hodgkin’s lymphoma derived from a subset of virgin B-cells localised in the mantle zone of primary follicles of peripheral lymphoid organs. They are histologically characterised by neoplastic expansion of the mantle zone, mimicking a nodular pattern or diffusely infiltrating adjacent lymphoid tissue. They account for less than 10% of all non-Hodgkin’s lymphomas in adults.

Mantle cell lymphoma is genetically characterised by t(11;14) translocation, an aberrant gene rearrangement between the IgH joining region (JH) on chromosome 14 and the bcl-1 locus on chromosome 11 at band 11 q13. This translocation is present in 60-70% of mantle cell lymphomas. The bcl-1 gene encodes the cell cycle-regulating protein cyclin D1. It spans 15 kb and has 5 exons and, despite chromosomal breaks, its sequence is not interrupted during the translocation, so that over-expression of cyclin D1 is produced in tumours that carry this gene. This over-expression plays a major pathogenic role in the growth of the tumour, probably dysregulating cell cycle control by overcoming the suppressor effect of retinoblastoma protein (Rb) and p27Kip1.

Numerous breakpoints in chromosome 11 are implicated in this translocation. Preferential breakpoints have been identified in the major translocation cluster (MTC), located 110Kb 5’ to bcl-1 gene, and, to a lesser extent, in the minor translocation clusters mTC1 and mTC2, which are located closer to the bcl-1 gene. Previous studies have shown that 80% of breakpoints are produced in the MTC region. Tests to detect MTC translocation by DNA amplification can detect most bcl-1 re-arrangements.

The classification of mantle cell lymphoma as a distinct entity was controversial for many years because of its heterogeneous morphology. Considering the relatively specific treatment of this type of tumour and given that its histological identification is difficult, the immunohistochemical identification of the malignant cells is mandatory (positivity for CD5 and cyclin D1). Nevertheless, diagnosis remains problematic in some cases so that molecular analysis of t(11;14) is required, above all when the primary study of the biopsy is not conclusive.

Because of its simplicity and high sensitivity, the use of DNA amplification to detect bcl-1 translocation offers new approaches to the diagnosis and treatment of the disease. In addition, this test allows study of the minimal residual disease in patients with mantle cell lymphoma and can aid assessment of the state of the disease and of the effects of therapy.

The fusion fragment between the bcl-1 gene and IgH is detected by DNA amplification using specific primers of MTC and JH sequences.

**KIT PRESENTATION**

It is a monotest kit with individual ready-to-use tubes that contain the reaction buffer, dNTP and specific primers. The amplification is carried out with specific primers that flank the MTC and JH regions using two nested amplification reactions, the second with primers that are internal to the first:
- 20 vials with mixture for the first amplification round M1-J1
- 20 vials with mixture for the second amplification round M2-J2, which generates a fragment with a size of 250-450 bp.

The kit also includes 20 tubes containing the reaction mixture to amplify a 274 bp fragment of tissue DNA corresponding to exon 5 of the p53 gene, which is used as an internal control to confirm the correct processing of the sample and the optimum quality of the DNA.

The result of the amplification can be visualised by electrophoresis in ethidium bromide-stained agarose gels.
PRESENTATIONS:
- 0.2 ml tubes  Cat. Nº: 003998M-2
- 0.5 ml tubes  Cat. Nº: 003998M-5

KIT COMPONENTS (20 DETERMINATIONS):

For amplification reaction:
- 1st amplification round M1-J1 mixture (Green tube): 20 vials x 46.5 µl
- 2nd amplification round M2-J2 mixture (Red tube): 20 vials x 48.5 µl
- Internal control mixture (Yellow tube): 20 vials x 46.5 µl
- DNA polymerase (2 U/µl): 1 vial X 60 µl
- Positive control DNA of Mantle cell lymphoma*: 20 µl

*NOTE: Repeated freezing and thawing of the DNA must be avoided. In order to prevent its degradation, the control DNA should be maintained at 4°C after its first thawing.

MATERIAL REQUIRED BUT NOT SUPPLIED IN THE KIT

Specific apparatus:
- Thermal cycler
- Microcentrifuge
- Bath/oven with thermostat
- Power supply
- Electrophoresis tray for DNA
- Ultraviolet light transilluminator
- Polaroid photography system

Disposable material:
- Xylene (or octane)
- 100% absolute ethanol
- Ficoll and PBS buffer (for isolation of lymphocytes from whole blood)
- DNase/RNase-free 1.5/0.6 ml Eppendorf tubes
- Reagents to carry out electrophoresis of the amplified DNA (agarose or polyacrylamide gels, TBE buffer, loading buffer, Ethium Bromide (EtBr), molecular weight marker). All of the reagents are included in the following Master Diagnostica Kits: DNA ELECTROPHORESIS IN AGAROSE GELS (Catalogue Nº 003980M).
- Reagents for DNA extraction from cell suspensions, fresh/frozen tissue or tissue sections fixed in buffered formalin and embedded in paraffin. All these are included in DNA EXTRACTION KIT Ref: MAD-003951M from Master Diagnostica.

CAUTION
The wearing of disposable gloves is recommended during the entire procedure. Because of the high sensibility of the DNA amplification method, it is recommended that the amplification reaction is performed using pipette tips with filter to avoid contamination. The major source of contamination is usually the amplified product itself, thus it is recommended that the handling and subsequent electrophoresis of the amplified products are done in a separate work area from where the samples are processed and that different pipettes are used in each case.

Transport and storage: The Kit is transported and stored at –20º C. Once the lysing solution and mineral oil have been thawed they can be kept at 4º C without having to be refrozen. The control DNA included in each kit must also be stored at 4º C when thawed.

This product is for in vitro diagnosis
PROTOCOL

1. EXTRACTION OF GENOMIC DNA

For DNA extraction it is recommend using the reagents included in the DNA EXTRACTION KIT (MAD-003951M). Before starting the DNA extraction, thaw out the reagents supplied in the kit: mineral oil, lysing solution and a vial of protease solution. After use, the mineral oil and the lysing solution can be stored at 4°C. The protease solution must be stored at -20°C, avoiding repeated freezing/thawing.

1.1. FROM PARAFFIN-EMBEDDED TISSUES

1. Take 2-6 tissue sections of 10 µm thickness (according to the amount of material in each section) and place in a 1.5 ml microcentrifuge tube using a needle or fine tweezers.
2. Dewax with 1 ml of xylene (or octane) and shake in vortex for 2-5 minutes until the paraffin is completely dissolved.
3. Centrifuge for 5 min at maximum speed in microcentrifuge. Carefully remove the supernatant without removing any tissue (it can be done with a capillary tube made from a Pasteur pipette).
4. Repeat steps 2 and 3.
5. Resuspend the pellet in 1 ml of 100% ethanol, shake and centrifuge for 2 min at maximum speed. Remove the supernatant and repeat the washing with ethanol two more times.
6. Remove well all ethanol remains and leave to dry in the air. The tissue must remain completely dry (to facilitate the drying it can be heated in an oven at 40-50°C). Once the tissue is dry, the samples can be kept at room temperature for several hours or days.

**Note:** the paraffin can also be removed by heating the sections in mineral oil using the following procedure:

Add 600 µl mineral oil, shake in vortex and heat in thermal cycler at 95°C for 2 min.
Centrifuge for 2 min at maximum speed.
Remove 480 µl mineral oil without removing tissue remains. Repeat the procedure.

7. Add 50 µl lysing solution and 1 µl protease solution (thawed at the time of use and kept in ice) to the resulting tissue pellet. Resuspend by agitating several times with the micropipette, centrifuge for 5 seconds to remove bubbles and incubate for 3 hours at 55°C with gentle shaking.

**Note:** Shaking during incubation increases the DNA performance, as does prolonging the incubation time to 24-48 h and adding 1µl protease solution every 12 hours. This prolonged incubation is recommended to obtain optimal results, above all when the tissue has not been fixed under ideal time or buffer conditions.

8. Heat at 95°C for 8-10 min to inactivate the protease.
9. Centrifuge for 5 min at maximum velocity and COLLECT THE SUPERNATANT (which contains the DNA), avoiding gathering tissue remains from the bottom of the tube. Use 3 µl of this DNA solution for the amplification. The sample can be stored at -20°C until its analysis and is stable for at least a week.

1.2 FROM FROZEN TISSUE

1. Cut several thin sections of frozen tissue using a scalpel blade and place them in a sterile 1.5 ml Eppendorf tube.
2. Add 50 µl lysis solution and 1 µl protease solution (thaw at the time of using and keep in ice). Resuspend by agitating several times with micropipette, centrifuge for 5 seconds to remove bubbles and incubate for 3 hours at 55°C with gentle shaking (the incubation can be prolonged to 24 h).
3. Heat at 95°C for 8-10 min to inactivate the protease.

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4. Centrifuge for 5 min at maximum speed and **COLLECT THE SUPERNATANT** (which contains the DNA), avoiding gathering cell remains from the bottom of the tube. Use 3 µl of this DNA solution for the amplification. The sample can be stored at –20ºC until its analysis and remains stable for a week.

### 1.3 FROM WHOLE BLOOD SAMPLES

**NOTE:** Do not use heparinised whole blood. EDTA or Sodium Citrate is recommended for use as anticoagulants.

1. Transfer 1.5 ml of total blood to a sterile 10-15 ml centrifuge tube with conical base.
2. Extract the lymphocytes by centrifugation in a Ficoll gradient following standard protocol:
   - Dilute the volume of blood with a similar volume of PBS buffer.
   - Place 2 ml of Ficoll in a 10-15 ml centrifuge tube with conical base
   - Add the diluted blood (3 ml) over the Ficoll, allowing it to fall slowly down the walls of the tube so that it does not mix with the Ficoll.
   - Centrifuge for 30 min at 900g at room temperature. At the end of the centrifugation, the lymphocytes are distributed in a whitish ring at the Ficoll – supernatant interface.
   - Aspirate with a Pasteur pipette the layer of lymphocytes at the Ficoll – supernatant interface and transfer to a 1.5 ml Eppendorf tube. The minimum volume of Ficoll and supernatant should be taken, because excess of the former produces contamination with granulocytes and excess of the latter produces contamination with plasma proteins.
   - Bring to 1.5 ml with PBS and centrifuge at half speed to precipitate the lymphocytes.
   - Repeat this last wash step.
3. Continue with the general DNA extraction protocol on the resulting lymphocyte pellet:
5. Add **50 µl lysis solution** and **1 µl protease solution** (thaw at the time of using and keep in ice). Resuspend by agitating several times with micropipette, centrifuge for 5 seconds to remove bubbles and incubate for **3 hours at 55ºC** with gentle shaking.
6. Heat at 95ºC for 8-10 min to inactivate the protease.
7. Centrifuge for 5 min at maximum speed and **COLLECT THE SUPERNATANT** (which contains the DNA), avoiding gathering cell remains from the bottom of the tube. Use 3 µl of this DNA solution for the amplification. The sample can be stored at –20ºC until its analysis and remains stable for at least a week.

### 2. AMPLIFICATION REACTION

**1st amplification round**

For each DNA sample two amplification reactions are performed in a first round:
- Reaction with M1-J1 rearrangement mixture (Green tube)
- Reaction with internal DNA control (Yellow tube)

Thaw one green tube and one yellow tube for each sample, keep in ice and add to each tube:

- 0.5 µl DNA-polymerase
- 3 µl DNA sample*

*If DNA of known, spectrophotometrically determined concentration is available, it is recommended that 300-500 ng of DNA is added.

Mix the tubes well, add 50 µl mineral oil (optional according to type of thermal cycler) and centrifuge for 5 seconds in microcentrifuge.

Note: It is important to keep the tubes in ice until the moment of their placing in the thermal cycler to avoid non-specific binding of the primers.
Place all the tubes in the thermal cycler and set the following programme:

Amplification conditions:

- 94°C 5 min
- 35 cycles:
  - 94°C 45 seconds
  - 60°C 45 seconds
  - 72°C 45 seconds
- 72°C 4 min

Keep the tubes refrigerated at 4°C when the reaction is finalised. If the samples are not to be processed immediately, they can be stored at –20°C.

The control DNA tubes (yellow) are reserved for the electrophoresis and the M1-J1 reaction mixture tubes (green) will serve as DNA template for the second round of amplification (nested).

**Note:** For 0.2 ml tubes the times in each cycle can be reduced as follows:

- 94°C 30 seconds - 60°C 30 seconds - 72°C 30 seconds.

### 2nd amplification round

For each DNA sample an amplification reaction is carried out in this second round with the M2-J2 rearrangement mixture (Red tube).

Thaw one red tube for each sample, keep in ice and add to each tube:

- 0.5 µl DNA-polymerase
- 1 µl of the PCR product for M1-J1 from 1st amplification round (Green tube)

Mix the tubes well, add 50 µl mineral oil (optional according to type of thermal cycler) and centrifuge for 5 seconds in microcentrifuge.

**Note:** It is important to keep the tubes in ice until the moment of their placing in the thermal cycle to avoid non-specific binding of the primers.

Place all of the tubes in the thermal cycler and set the following programme:

Amplification conditions:

- 94°C 5 min
- 20 cycles:
  - 94°C 45 seconds
  - 55°C 45 seconds
  - 72°C 45 seconds
- 72°C 4 min

Keep the tubes refrigerated at 4°C when the reaction is finalised. If the samples are not to be processed immediately, they can be stored at –20°C.

**Note:** For 0.2 ml tubes, the times in each cycle can be reduced as follows:

- 94°C 30 seconds - 55°C 30 seconds - 72°C 30 seconds.
2. ELECTROPHORESIS OF THE AMPLIFIED PRODUCTS:

Caution: given the high sensitivity of the amplification technique, which generates large amounts of a specific DNA fragment, the amplified product is a powerful source of contamination in the laboratory. It is recommended that the handling and electrophoresis of the amplified products is done in a separate work area from where the sample processing is done, to avoid contamination with the amplified DNA, which could lead to false positive diagnoses.

The amplified products can be developed in agarose (3%) gel with TBE 1X buffer. Master Diagnostica offers Kits for DNA electrophoresis in ready-to-use agarose gels that also include all the reagents required for the electrophoresis: molecular weight marker, loading buffer, concentrated TBE buffer, EtBr (Catalogue N°: 003980M).

PROCEDURE:
For each DNA sample, the amplified products of the red and yellow tubes, corresponding to the 2"nd amplification of the bcl-1 gene (M2-J2) and the amplification of the internal control DNA, are analysed by electrophoresis.

Take 20 µl of the amplified product, transfer to an Eppendorf tube and add 4 µl 6X loading buffer. Load the wells with the samples and place in one of the lanes 10 µl of the molecular weight marker. Run the electrophoresis for 1 hour at 100 volts. Stain with EtBr and visualise on ultraviolet transilluminator.

3. RESULTS INTERPRETATION

INTERNAL CONTROL

Amplification of exon 5 of the p53 gene (Yellow tube):

In all cases an intense band of 274 base pairs should appear, indicating correct sample handling and optimum DNA quality.

If the DNA control fails, there are two possibilities:
1. The tissue was not adequately fixed, so that the performance and quality of DNA are very low. In this case the DNA quality can be improved by prolonging the incubation of the tissue with the lysis buffer for up 48 hours. DNA quality can also be improved by subsequent purification with phenolic extraction and precipitation or by the use of similar commercial Kits.
2. The amount of original material is too small. If the sample is very small (<2mm²), the test should be repeated, increasing the number of paraffin block or frozen tissue sections.

t(11;14) translocation

The t(11;14) translocation fuses the bcl-1 locus on chromosome 11 with the IgH joining region (JH) on chromosome 14 and is present in 60-70% of mantle cell lymphomas. The use of primers targeting the MTC sequence allows detection of most bcl-1 rearrangements because 80% of the break-points occur in this region.

If this translocation has been produced, a single band 250 - 450 bp in size should appear after amplification using primers that flank the translocated region of each chromosome (11 and 14). If no translocation has occurred, these primers will hybridise to the two distinct chromosomes and the amplified product will not be obtained.
4. REFERENCES


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