IgH REARRANGEMENTS MOLECULAR ANALYSIS KIT

CAT. Nº MAD-003994BP-2/5 (20 DETERMINATIONS)

The diagnosis of malignant lymphomas is one of the most challenging tasks in histopathology. While many cases can be diagnosed from histomorphological and immunohistochemical data, it is occasionally difficult to distinguish between a reactive process and a malignant lymphoma. In these situations, detection of clonality using molecular analysis by PCR of the rearrangement of immunoglobulin (Ig) and TCR genes is highly valuable for diagnosing B and T lymphoproliferative processes. Rearrangements in the Ig and TCR genes give rise to hypervariable regions, and each mature lymphocyte has a specific rearrangement in these regions with a unique sequence and length. For this reason, if DNA from a normal or a reactive lymphoid population is amplified, the result will be multiple fragments within a specific range of sizes and following a Gaussian distribution. When DNA is amplified from a clonal lymphoid population, the resulting fragments are all identical in size and sequence, giving a band or single major peak.

This kit allows detection of clonality in B cell lymphoproliferative processes by amplification of rearranged VDJ segments in the hypervariable region of the immunoglobulin heavy chain (IgH), using multiple primers that hybridise with conserved regions in the genes.

Kit characteristics

- Contains an amplification mix for each one of the segments FR1-JH, FR2-JH and FR3-JH, and an internal control for verifying DNA quality.

-The amplification mixtures are in “monotest” form in colour-identified PCR tubes of 0.2 - 0.5 ml.

- All the mixtures include primers labelled at the 5’ end with 6-FAM fluorochrome, which allows automatic analysis of fragments by capillary electrophoresis using GeneScan.

-All amplifications can be carried out in the thermocycler using a single programme.

-The enzyme Phire® Hot Start II DNA Polymerase is supplied in the kit.

-Positive controls for clonal and polyclonal DNA are included.

This product is for in vitro diagnostic

Components included in the kit:

Table1. Composition of reagents:

REAGENT	REFERENCE	COMPONENTS	QUANTITY
Amplification mixtures (monotest in tubes of 0.2 or 0. 5 ml)	MAD-003994BP-2/-5	FR1-JH (6-FAM) (mix of oligonucleotides from regions FR1 and JH of IgH gene, dNTP and buffer solution)	20 PCR tubes:red (46 µl)
		FR2-JH (6-FAM) (mix of oligonucleotides from regions FR2 and JH of IgH gene, dNTP and buffer solution)	20 PCR tubes:green (46 µl)
		FR3-JH (6-FAM) (mix of oligonucleotides from regions FR3 and JH of IgH gene, dNTP and buffer solution)	20 PCR tubes:purple (46 µl)
		Internal control mix (CI) (6-FAM) (oligonucleotides specific for exon 5 of p53 gene, dNTP and buffer solution)	20 PCR tubes:yellow (46 µl)
DNA polymerase	MAD-F122-2	Phire® Hot Start II DNA Polymerase*	2 x 60 µl
Positive control DNAs	MAD-003994B2	Clonal B positive control DNA (50 µg/ml)	1 x 50 µl
	MAD-003994B3	Polyclonal positive control DNA (100 µg/ml)	1 x 50 µl

* ***Trademark and license statements***

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Necessary equipment and materials not supplied in the kit:

Equipment:

Thermocycler
Microcentrifuge
Thermostatic bath or heating block
Power supply
Electrophoresis tray for DNA
UV transilluminator
Gel documentation system
Capillary electrophoresis sequencing equipment

Consumables:

Xylene (optional)
100% Ethanol
PBS buffer (for extraction of lymphocytes from whole blood)
TE buffer 1x (10 mM Tris, 1 mM EDTA pH 8.0)
Eppendorf tubes 1.5/0.6/0.2 ml, DNase/RNase-free
Reagents for electrophoresis of amplified DNA (agarose or polyacrylamide gels and auxiliary reagents). *All these are included in the* following ***Master Diagnóstica*** kits***: ELECTROPHORESIS OF DNA IN AGAROSE and POLYACRYLAMIDE GELS Refs: MAD-003980M and MAD-003990M*** *respectively****.***
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.***
Reagents for fragment analysis by GeneScan: POP-4 Polymer, molecular weight standard GeneScan 400HD ROX, EDTA buffer and deionised formamide

Precautions:

The wearing of disposable gloves is recommended throughout the entire procedure.

Because of the high sensitivity of the DNA amplification method, it is recommended that the amplification reaction is performed using pipette tips with filters to avoid contamination.

The main source of contamination is usually the amplified product itself, hence it is recommended that the handling and subsequent electrophoresis of the amplified products are done in a work area separate from that where the samples are processed and that different pipettes are used in each case.

Three work areas should be established: an area for processing and preparation of DNA samples, an area for amplification and an area for detection (electrophoresis). The work flow must always be in the same direction, from the preparation zone through the amplification zone to the detection zone, *never in the opposite direction*.

It is recommended to include negative controls for amplification, containing all reagents supplied in the kit except for the DNA samples. This allows detection and control of any contamination of the reagents with DNA resulting either from problems with the samples or from amplified products.

Transport and storage:

The Kit is transported and stored at –20ºC. Once the lysing solution has been thawed it can be kept at 4ºC without having to be refrozen. Once thawed, the control DNAs in each kit can also be stored at 4ºC.

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 4ºC. The protease solution must be stored at -20ºC, avoiding repeated freezing/thawing

1.1. SECTIONS OF PARAFFIN-EMBEDDED TISSUES

1. Take 1-4 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. Add **500 µl** of **mineral oil** (included in the kit) heat in a heating block for **2 min at 95ºC**. Centrifuge 2 min at 8000 rpm in the microcentrifuge.
3. Remove the oil without disturbing the tissue fragments.
4. Repeat steps 2 and 3. (The remaining mineral oil does not interfere with the DNA extraction process).
5. Prepare a **50:1** mixture of **lysing** and **protease solutions** (for every **50 µl** of lysing solution add **1 µl** of protease solution) in sufficient volume for processing the tissue samples.
6. To the resulting tissue pellet add an **adequate volume (50-500 µl) of the mixture above** until the tissue is fully in suspension (this is important to ensure a good yield of DNA and degrading of contaminating cell remains which could interfere with subsequent amplification of this DNA).
7. Agitate several times using the micropipette to homogenize, centrifuge for 5 sec to remove bubbles and incubate for **24-48 h** at **55ºC in** thermostatic bath or heating block.
8. Heat at **95ºC** in the thermal block for **8-10 min** to inactivate the protease.
9. Centrifuge for 5 min at maximum speed. After centrifugation there should be 2 phases, the upper containing the remains of the mineral oil and lower aqueous layer that contains the dissolved DNA. There may be a small pellet of undigested tissue at the bottom of the tube. **COLLECT THE AQUEOUS PHASE** containing the DNA, avoiding any tissue remains at the bottom of the tube.
10. Use **3 µl** of this DNA solution for amplification. The sample can be stored in this state, being stable at 4ºC for 1 week, or at -20/-80ºC for several months.

1.2. FROZEN TISSUE

1. Cut 1-2 sections of tissue in the cryostat or using a scalpel blade and place in a 1.5 ml microcentrifuge tube using a needle or fine tweezers.
2. Continue with **step 5** of the previous protocol for paraffin sections.

Note: *To avoid evaporation of the lysing/protease solution during the incubation at 55ºC, a few drops of the mineral oil included in the kit can be added.*

1.3 PERIPHERAL BLOOD OR BONE MARROW SAMPLES

Note: *Do not use heparinized whole blood. We recommend the use of EDTA or sodium citrate as anticoagulants.*

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1. Isolate the white cell population using the **Buffy Coat** from **1.5 ml** of **blood/marrow** with EDTA. Fractionate by centrifuging at **1500-2000 x g** for **10-15 min** at room temperature. This procedure separates an upper phase of plasma, a lower phase with the red cell population and a thin interphase with white cells (the buffy coat). (For a typical clinical centrifuge, 1500-2000 x g is equivalent to 3000-3400 rpm).

2. Collect the white cell population and place it in a clean tube.

3. Wash with **5 ml** of **TE 1X** buffer and incubate for **10 min at 37ºC** to lyse any remaining haemocytes.

4. Centrifuge at **3000 rpm** for **5 min** to precipitate white cells.

5. Re-suspend the cell pellet in **1 ml** of **TE 1X** buffer and transfer to a 1.5 ml Eppendorf tube.

6. Centrifuge at **8000 rpm** for **5 min** in a microcentrifuge and discard the supernatant.

7. Continue with **step 5** of protocol 1.1 for paraffin-embedded samples.

Note: *To avoid evaporation of the lysing/protease solution during the incubation at 55ºC, a few drops of the mineral oil included in the kit can be added.*

2. AMPLIFICATION REACTION

For each analysis sample of test DNA, four mixtures are amplified: one for each of the fragments FR1, FR2 and FR3 and an internal control mix. The mixes should be protected from light since they contain fluorescence-labelled primers.

2.1. SAMPLE PREPARATION

1. Thaw 1 **red** tube **(FR1-JH),** 1 **green** tube **(FR2-JH),** one **purple** tube **(FR3-JH)** and one **yellow** tube **(internal control)** for each sample, and, keeping them on ice, add the following to each tube:

   - 1 µl of enzyme Phire® Hot Start II DNA Polymerase
   - **3 µl** of the **DNA sample*****

2. Mix well and centrifuge for 5 sec in the microcentrifuge to remove bubbles.

   ***If the DNA is of known concentration, 200-500 ng of DNA per tube is recommended.

Note: *It is important to keep the samples on ice until they are placed in the thermocycler to avoid non-specific binding of the primers.*

2.2. AMPLIFICATION

1. Place all tubes in the thermocycler and amplify using the following programme:

98ºC 2 min.
35 cycles:
98ºC 10 s
60ºC 10 s
72ºC 15 s
72ºC 1 min

2. When the reaction is over, keep the tubes at 12-15ºC. If the samples are not to be processed immediately, they can be stored at 4ºC or -20ºC until use.

2.3. RECOMMENDED AMPLIFICATION CONTROLS

The kit supplies **clonal and polyclonal positive control DNAs,** which should be amplified with each batch of samples analyzed.

To control possible cross-contamination between samples or contamination of the common reagents used during sample handling, we recommend **the processing of a blank sample** without DNA. This contains all the same reagents employed in the DNA extraction from samples and is prepared using the same processing procedures. Because the blank sample contains no DNA, it should give a negative result for all three mixtures after amplification.

Likewise, although not essential, it is advisable to analyze each sample in duplicate, i.e., dividing the same genomic DNA to **carry out the amplifications** of all fragments **in duplicate.** This facilitates interpretation of doubtful results and confirms, using the duplicate, the size of monoclonal peaks in the profile of a reactive polyclonal population.
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3. ANALYSIS OF AMPLIFIED PRODUCTS

3.1. GEL ELECTROPHORESIS

WARNING! Given the high sensitivity of the amplification technique, which generates large quantities of a specific DNA fragment, this amplified product represents a potent source of contamination in the laboratory. It is recommended that the handling and electrophoresis of amplified products be carried out in a work area well removed from the sample preparation area to avoid contamination by amplified DNA and consequent false positive results.
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Amplified products can be visualised by using 4% agarose or 6-8% polyacrylamide gels with half-strength TBE buffer. Polyacrylamide gels give the best resolution.

Master Diagnóstica supplies “ready-to-use” kits for DNA electrophoresis in either agarose or polyacrylamide gels, including all reagents needed for electrophoresis: molecular weight marker, loading buffer, 0.5 x TBE buffer concentrate and EtBr (**Cat. Nº MAD-003980M and MAD-003990M for agarose and polyacrylamide, respectively**).

Heteroduplex analysis of the PCR products is recommended to differentiate between products derived from mono and polyclonal populations. If there has been a clonal rearrangement of the IgH genes, a “homoduplex” will be obtained, whereas in a polyclonal population, a “heteroduplex” with heterogeneous binding will be formed after the denaturing/renaturing procedure.

3.1.1. PROCEDURE

1. Denature the PCR products at **94ºC** for **5 min**.
2. Renature by transferring rapidly onto ice (4ºC) for 10-60 min.
3. Assemble the agarose or polyacrylamide gel in its tray and cover with electrophoresis buffer TBE 0.5X.
4. Take **20 µl of PCR product** and mix with **4 µl** of **loading buffer 6X**.
5. Load the samples into the wells in the gel, placing **10 µl** of **molecular weight marker** in one of the tracks.
6. Carry out electrophoresis for **1-2 h at 100 V**. Voltage and running time can vary depending on the type of gel, the electrophoresis tray, and the size of the amplified product, among other factors.
7. Stain with **0.5 µg/ml EtBr** in water or TBE 0.5X and visualize with a UV transilluminator.

3.2. CAPILLARY ELECTROPHORESIS

PCR mixes FR1-JH, FR2-JH, FR3-JH and internal control mix, contain primers labelled with 6-FAM fluorochrome. In addition to visualization by conventional electrophoresis, this allows the PCR products to be analyzed using capillary electrophoresis in GENESCAN.

Automatic sequencers based on capillary electrophoresis, e.g., ABI PRISM® 310 and 3100 Genetic Analyzers from Applied Biosystems, are systems with high levels of reproducibility and sensitivity in the sequencing and analysis of genomic fragments. Their performance is superior to that of the majority of sequence analyses based on the use of polyacrylamide gels. Moreover, these systems allow rapid and accurate analysis of the results using specific software.

3.2.1. PREPARATION OF THE PCR PRODUCT

1. Mix in a 0.2-ml Eppendorf tube:
   15 µl of deionised formamide + 0.5 µl of 400HD ROX marker + 1 or 2 µl of amplified DNA (this volume of amplified product can be changed if the fluorescent signal is outside the optimum range).
2. Homogenize with a pulse of the vortex mixer.
3. Denature at **95ºC** for **5 min**, chill to 4ºC and load into the sequencer.

Electrophoresis conditions:

Gel: 3100 POP4 polymer

Buffer: 3100 buffer with EDTA 1X

Electrophoresis: approx. 25-30 min.

Optimum fluorescence intensity: up to a maximum of 10,000 units

3.2.2. OPTIMUM CONDITIONS FOR SAMPLE LOADING

To test whether the DNA sample has been processed correctly and is of adequate quantity and quality to generate a valid result in the electropherogram, we recommended loading an aliquot of the PCR products (including the internal control) and carrying out conventional electrophoresis with EtBr staining before the GeneScan analysis.

An excessive load of PCR product in capillary electrophoresis can lead to artefact peaks for the molecular weight standard and the product, leading to a false reading. Thus, excess of a clonal sample can produce peaks that resemble the pattern for a polyclonal sample.

To avoid this problem, the fluorescent signal should be kept between 400 and 7000 fluorescence units in models 310 and 3100.

4. RESULTS INTERPRETATION

4.1. INTERNAL AMPLIFICATION CONTROL (CI)

An intense band or a peak (GeneScan) of **274 base pairs** should appear in all samples, indicating that the sample handling and DNA quality were adequate. This amplification control is essential, especially for samples from paraffin-embedded tissues, in which the quantity and quality of the DNA obtained are unknown.

If no band/peak appears after successful amplification of the internal control, a negative result for the amplification of IgH fragments cannot be assumed, since inadequate DNA may have been extracted or it may have partly degraded. In these cases, the yield and quality of the DNA can be improved by prolonging incubation of the tissue with lysing solution + protease for a further 24-48 h.

If no amplification is obtained after repeating this procedure, the sample is considered “non-valid for analysis of IgH gene fragments by PCR”.

4.2. REARRANGEMENT OF IgH FRAGMENTS

The following table shows the expected size range of amplified fragments for each amplification mix:

Table 2.

Primer mix	*Size range*	*Clonal positive control DNA*	*Colour (PCR tube)*
FR1-JH	310-360 bp	338 bp	Red
FR2-JH	250-295 bp	274 bp	Green
FR3-JH	100-170 bp	133 bp	Purple

4.2.1 POSITIVE AND NEGATIVE AMPLIFICATION CONTROLS:

Negative amplification control with a **blank sample** should not show amplification in any of the mixes tested (including internal control). If amplification is detected in any PCR mix, this indicates reagent contamination, and a repeat test is required to confirm the result.

The **positive polyclonal control** should give multiple bands (gel) or peaks (GeneScan) within the size range shown in Table 2 for each amplification mix.

The **clonal positive control** should give a single band or peak for each PCR test mix. Sizes are shown in Table 2.

If analysis of the positive control DNAs shows a different result from the above, the test is invalid and the test sample cannot be interpreted.

4.2.2 ANALYSIS OF THE TEST SAMPLE

Before interpreting results obtained with the test sample, all positive and negative controls included in the test must have given a correct result.

AGAROSE OR POLYACRYLAMIDE GELS

In the electrophoresis study, clonal rearrangement of the IgH gene is shown by the presence of a single strong, sharp band within the expected size range (Figure 1). **A sample is considered "positive" when a band with these characteristics is detected with either or both of the two test amplification mixes: FR1-JH, FR2-JH o FR3-JH.**

When there is no clonal rearrangement and the test sample comprises a heterogeneous polyclonal population of B cells, a heteroduplex will be formed, and electrophoresis will reveal **multiple bands within the size range, visualized on the gel as a broad and diffuse stain or smear (Figure 1).**

CAPILLARY ELECTROPHORESIS

The amplification products marked with fluorochrome are separated by capillary electrophoresis as a function of size and detected automatically with a laser.

In the case of a polyclonal lymphoproliferative process, the result (Figure 2) can be a Gaussian distribution with multiple peaks, representing multiple amplification products of different sizes (within the expected size range), and it should be reported that **"the analyzed sample shows no clonal rearrangement for IgH gene under the conditions of the test".**

When there are one or two single or prominent peaks in the size range with one or two of the primer mixes tested, this corresponds to a clonal lymphoproliferative process (Figure 2). The report would be

that "**the sample analyzed shows clonal rearrangement for the IgH gene under the conditions of this test**".

The electropherogram can sometimes show multiple peaks of different sizes, with one or more that are higher than the rest. The criterion for deciding whether one or more peaks are positive is that they are **at least 2.5-fold higher than adjacent peaks that represent the polyclonal background**. I**f an amplification duplicate has been produced, the finding of a peak of equal size in the duplicate also helps to identify it as positive**. If no peaks reach this relative height, they are all considered to be part of a polyclonal population. If peaks reach the minimum height to be considered clonal but are not of the same size in the duplicate, they should be considered pseudo-clonal peaks, resulting from amplification of a DNA sample containing a small number of B cells from within a benign lymphoproliferative process.

Figure 1. **Analysis of results in 4% agarose gel. A. Mix FR1-JH:** 1. MW standard, 2. monoclonal B sample, 3. monoclonal B sample, 4. monoclonal B sample, 5. polyclonal B sample, 6. monoclonal B sample, 7. B cell line, 8. tonsil. **B. Mix FR2-JH:** 1. MW standard, 2. B cell line, 3. monoclonal B sample, 4. monoclonal B sample, 5. polyclonal B sample, 6. polyclonal B sample, 7. polyclonal B sample, 8. tonsil. **C. Mix FR3-JH:** 1. MW standard, 2. tonsil, 3. B cell line, 4. monoclonal B case, 5. polyclonal B case, 6. monoclonal B case, 7. polyclonal B case.

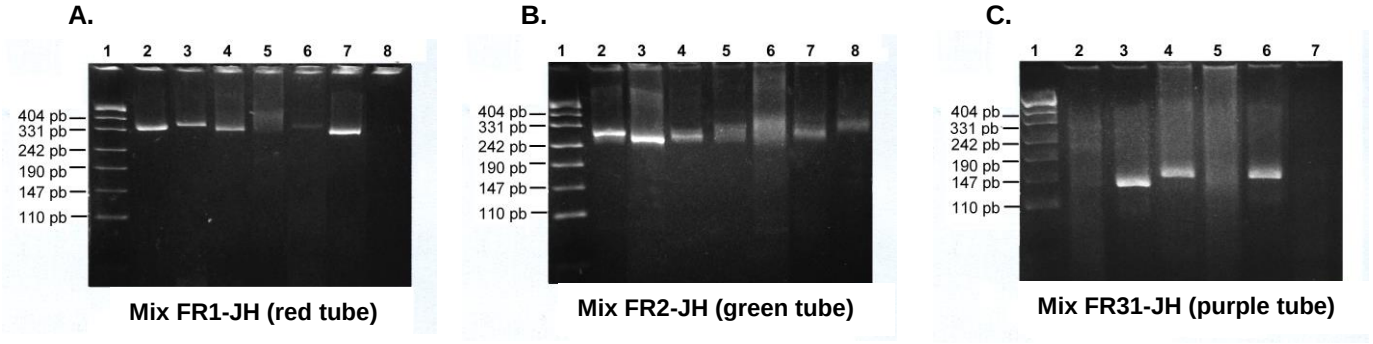
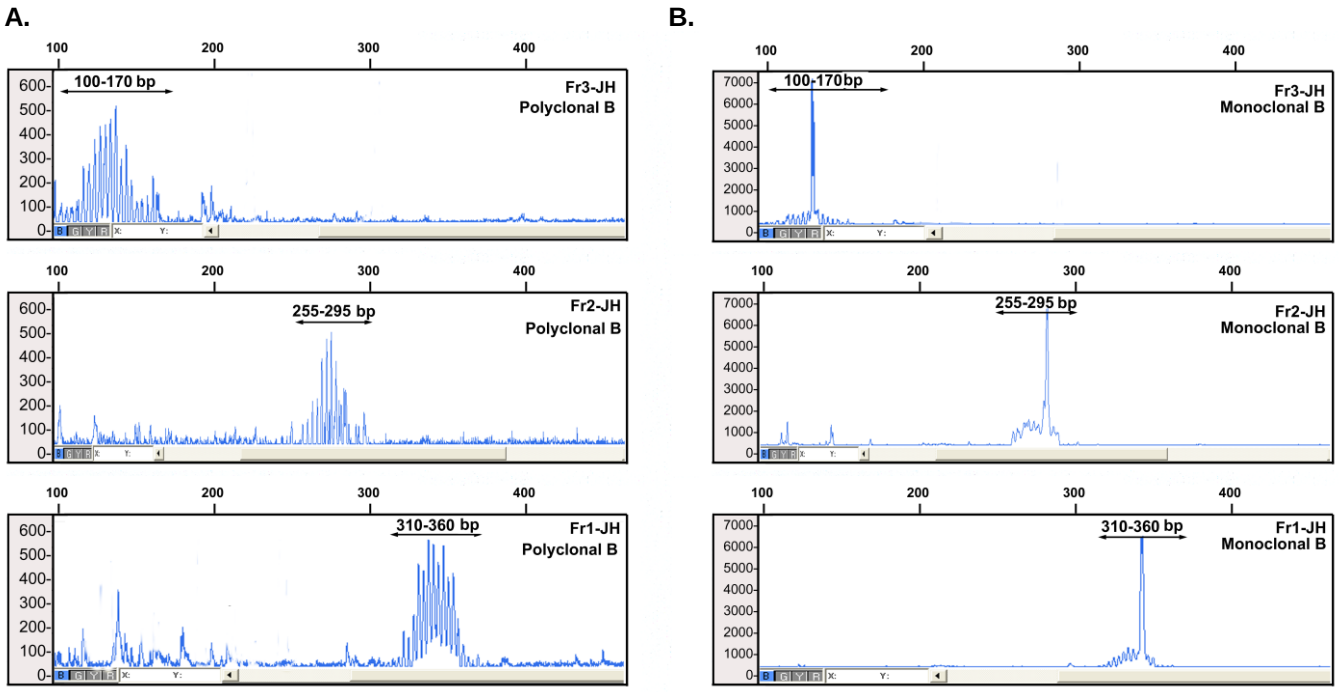


Figure 2. Results of GeneScan capillary electrophoresis A. Typical Gaussian distribution of a polyclonal population obtained with PCR products FR3-JH, FR2-JH and FR1-JH. **B.** Monoclonal peaks obtained with PCR products FR3-JH, FR2-JH and FR1-JH.



5. TEST LIMITATIONS

In a sample containing a mixture of tumour (clonal) and reactive B-cell populations, the sharp band indicating cloning may be masked by other multiple bands and may not be visualised by either gel electrophoresis or GeneScan.

The method has the capacity to detect 1.5 - 2 tumour cells in 100 normal cells (Table 3). This result was obtained by testing the three amplification mixtures with serial dilutions of a DNA sample from a lymphoma B cell line (RAMOS) with DNA from peripheral blood lymphocytes.

Table 3. *Limits of detection*

Mix	***Limit of detection***
FR1-JH	4 ng clonal DNA (2 % in 0.2 µg total)
FR2-JH	4 ng clonal DNA (2 % in 0.2 µg total)
FR3-JH	3 ng clonal DNA (1.5% in 0.2 µg total)

Monoclonal amplification combining the three primer mixes (FR1-2-3/J) in this kit is specific for the B line, diagnosing about 90% of all mature B-cell lymphoproliferative processes. A negative result for cloning by this test does not, of course, rule out a diagnosis of lymphoma based on other clinical, morphological or immunophenotypic findings.

6. REFERENCES

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