TCR Gamma Rearrangements Molecular Analysis Kit

CAT. No. MAD-003994TP-2 (20 tests)

The diagnosis of malign lymphomas is one of the most difficult areas in histopathology. Although many cases are diagnosed through histomorphological and immunohistochemical data, occasionally, the differential diagnosis between a reactive process and a malign lymphoma is difficult to determine. In these cases, the detection of clonality through molecular analysis by PCR of rearrangements of immunoglobulin (Ig) and TCR genes is an instrument of great value in the diagnosis of lymphoproliferative processes B and T. The rearrangements for Ig and TCR occur in the hypervariable regions of these genes. Each mature lymphocyte presents a specific rearrangement with a single length and sequencing in these regions. Therefore, if what is amplified is the DNA of a normal or reactive lymphoid population, the result will be multiple fragments within a given size range, with a Gaussian distribution. When amplifying DNA from a tumor and clonal process, all the resulting fragments will be identical in sequence and size, obtaining a single majority band or peak.

The TCRgamma gene is a preferential target for the analysis of T clonality, since it rearranges very early during the T lymphoid development, probably right after the TCRdelta, both in the TCR precursors$\gamma\delta$ and TCR$\alpha\beta$.

This kit makes it possible to detect the presence of clonality in T-source lymphoproliferative processes by amplifying the rearranged VJ segments of the TCRgamma gene. Given the diversity of sequences of these TCR genes, multiple targeted primers are used against conserved regions flanking V and J segments in order to detect as many clonal rearrangements as possible.

Characteristics of the kit:

- It contains two mixes of multiplex reaction (Mix A and Mix B) of the V-J region of the TCRgamma gene and an internal amplification control (IC) to check the DNA quality.
- The amplification mixes are presented in mono-test format in PCR tube strips of 0.2 ml, identified in different colors.
- All PCR mixes include 5' end labeled primers with fluorochrome 6-FAM, allowing automatic analysis of fragments by capillary electrophoresis (compatible with ABI PRISM® 310, 3100, 3130, 3130xl, 3500 and 3500xL Genetic Analyzers).
- All amplifications can be performed with a single program in the thermal cycler.
- The Hot Start II DNA Polymerase is enzyme is provided in the kit.
- Clonal and polyclonal DNA positive controls are included.

This is a product for in vitro diagnostics

Components included in the kit:

Table 1. Composition of reagents:

REAGENT	REFERENCE	COMPONENTS	AMOUNT
Amplification mixes (mono-test in tubes of 0.2)	MAD-003994TP-2	VJ-A (6-FAM) (mix A of oligonucleotides from regions V_γ and J_γ of the TCRgamma gene, dNTP and buffer solution)	20 blue-colored PCR tubes (46 µl)
		VJ-B (6-FAM) (mix B of oligonucleotides from regions V_γ and J_γ of the TCRgamma gene, dNTP and buffer solution)	20 purple-colored PCR tubes (46 µl)
		Mix of Internal Control (IC) (6-FAM) (gene specific oligonucleotides p53 exon 5, dNTP and buffer solution)	20 yellow-colored PCR tubes (46 µl)
DNA polymerase	MAD-F122-1	Hot Start II* DNA polymerase	1 x 80 µl
DNA positive controls	MAD-003994T2	DNA clonal positive control T	1 x 50 µl
	MAD-003994B3	DNA polyclonal positive control	1 x 50µl

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Phire DNA Polymerase is manufactured by Thermo Fisher Scientific. PhireTM is a trademark or registered trademark of Thermo Fisher Scientific, Inc. or its subsidiaries.

Necessary material not provided in the kit:

- **Specific instrumentation:**
  - Thermal cycler
  - Microcentrifuge
  - Thermostatic bath / heater
  - Power supply
  - DNA electrophoresis chamber
  - UV Transilluminator
  - Gel documentation system
  - Sequencer with capillary electrophoresis

- **Consumables:**
  - Xylene (optional)
  - Ethanol 100%
  - PBS buffer (for the extraction of lymphocytes from whole blood)
  - TE buffer 1x (10 mM Tris, 1 mM EDTA pH 8.0)
  - DNase/RNase-free Eppendorf tubes of 1.5/0.6/0.2 ml

Reagents for the extraction of genomic DNA from samples of blood, cell buttons, fresh tissues, or formalin-fixed paraffin-embedded tissues. All the reagents are included in ***Vitro S.A.'s kit: PARAFFIN TISSUE PROCESSING KIT Ref: MAD-003952M.***

Reagents for electrophoresis of amplified DNA (agarose or polyacrylamide gels and auxiliary reagents for electrophoresis). *All the reagents are included in* ***Vitro S.A. S.L.'s Kits: Agarose Gel DNA Electrophoresis Kit*** and ***Polyacrylamide Gel Electrophoresis Kit Ref: MAD-003980M and MAD-003990M*** *respectively.*
Reagents for analysis of fragments by capillary electrophoresis (adjust according to the filter/equipment used; *ABI PRISM®* 310, 3100, 3130, 3130xl, *3500* and *3500xL* Genetic Analyzers): standard polymer of molecular weight, Cathode and Anode buffers and deionized formamide.

Precautions:

It is advisable to use disposable gloves during the whole development of the technique.
Due to the high sensitivity of the DNA amplification technique, it is recommended that the amplification reaction is carried out using filtered pipette tips to avoid contamination.
The major source of contamination is usually the amplified product itself, so it is advisable to carry out the handling of the amplified products and the subsequent electrophoresis in working areas separate from the area where the samples are processed and to use different pipettes in each case. Three working areas should be defined: the DNA sample processing and preparation area, the amplification area and the detection area (electrophoresis). The workflow must always go in a single direction, from the sample preparation and amplification area to the detection area, and never in the opposite direction.
It is recommended to include negative amplification controls containing all the reagents handled in the kit, except for the DNA sample, in order to detect and control any possible contamination of the reagents with DNA both from test samples and from amplified products.

Storage and transport:

The kit is transported under cooling conditions (-10/-30°C or 2-8°C) and is stored at -20°C. The control DNA included in every kit must be stored at 2-8°C after thawing.

Safety Recommendations

The product is only intended for professional laboratory purposes, and it is not intended for pharmacological, home or any other type of use. The current version of the material safety data sheet of this product can be downloaded by searching its reference at www.vitro.bio or can be requested at regulatory@vitro.bio.

TECHNICAL PROCEDURE

1. EXTRACTION OF GENOMIC DNA

For the DNA extraction it is recommended to use the reagents included in the ***PARAFFIN TISSUE PROCESSING KIT (Ref: MAD-003952M)***.
Before beginning with the DNA extraction, thaw the reagents supplied in the kit: mineral oil, extraction buffer and a vial of DNA release. After use, the mineral oil must be stored at 15-25 °C and the rest of the components must be kept at -20 °C.

1.1. PARAFFIN-EMBEDDED TISSUE SECTIONS

1. Take 1-4 tissue sections (according to the amount of material present in each section) of 10 µm thick and put them in a microcentrifuge tube of 1.5 ml with the aid of a needle or thin tweezers.

2. Add **500 µl** of **mineral oil** (included in the kit) and heat on hot plate for **2 min at 95°C**. Centrifuge for 2 min at 8000 rpm in microcentrifuge.

3. Remove the mineral oil without dragging tissue debris.

4. Repeat steps 2 and 3. (Mineral oil residues do not interfere in the process of DNA extraction).

5. Prepare a mix of **Extraction buffer and DNA release** (for every **60 µl** of extraction buffer, add **1.5 µl** of DNA release) in enough volume to process the tissue samples.

6. **Add an adequate volume of the previous mix (50-500 µl)** to the resulting tissue button until the tissue becomes suspended in the solution (this step is very important to achieve a good DNA performance and degrade the contaminant cell debris that may interfere in the subsequent amplification of this DNA).

7. Shake several times with a micropipette to homogenize, centrifuge for 5 seconds to eliminate the bubbles and incubate for **24-48 hours** at **60°C** in thermostatic bath or hot plate.

8. Heat at **95º C** on hot plate for **8-10 min**.

9. Centrifuge for 5 min. at full speed. After centrifuging, two phases should remain: an upper phase corresponding to the remains of mineral oil and a lower aqueous phase containing the DNA in solution. On the bottom of the tube, a small button with non-digested tissue debris must remain. **COLLECT THE AQUEOUS PHASE** (it contains the DNA) avoiding taking tissue debris from the tube's bottom.

10. Use **3 µl** of this DNA solution to amplify. The sample can be stored, being stable at 4 °C for a week, or at -20/-80 °C for several months.

1.2. FROZEN TISSUE

1. Prepare 1-2 tissue sections in cryostat or by means of a scalpel blade and put them in a microcentrifuge tube of 1.5 ml with the aid of a needle or thin tweezers.
2. Continue with **step 5** of the previous protocol for paraffin-embedded sections.

Note: *In order to avoid evaporation of the extraction buffer mix and DNA release during incubation at 60 °C, a few drops of mineral oil, included in the kit, may be added.*

1.3. PERIPHERAL BLOOD/BONE MARROW SAMPLES

Note: *Do not use heparinized whole blood. It is recommended to use EDTA or Trisodium Citrate as anticoagulant.*

1. Isolate white population with **Buffy Coat**: use **1.5 ml** of **blood/marrow** with EDTA and divide by centrifuging at **1500-2000 x g** for **10-15 min** at room temperature. This process will split an upper phase of plasma, a lower one with red population and a thin interphase between the two with white cells (buffy coat). (In a typical clinical centrifuge 1500-2000 x g equals to 3000-3400 rpm).

2. Collect the intermediate white population and transfer to a clean tube.

3. Wash **with 5 ml** of **TE buffer 1X** and incubate for **10 min at 37º C** to lyse the rest of haematids.

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

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

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

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

Note: *In order to avoid evaporation of the extraction buffer mix and DNA release during incubation at 60 °C, a few drops of mineral oil, included in the kit, may be added.*

2. AMPLIFICATION REACTION

For each test DNA sample to analyze, 3 mixes will be amplified: mixes VJ-A and VJ-B of the TCRgamma gene and mix of internal control (IC). It is recommended to preserve all the mixes of the light, since they contain primers labeled with fluorescence.

2.1. SAMPLES PREPARATION

1. Thaw in a **blue** tube **(VJ-A)** a **purple** tube **(VJ-B)** and a **yellow** tube **(IC)** for each sample (the tube and cap strips are divisible by sectioning). Centrifuge briefly and keep in ice. Add to each of them following this order:

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

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

*****If DNA of known concentration is available, it is recommended to add between 50-200ng of DNA.

Note: *It is important to keep the tubes in ice up to the moment of placing them in the thermal cycler in order to avoid non-specific binding of primers.*

2.2. AMPLIFICATION

1. Place all the tubes in the thermal cycler and amplify according to the program below:

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

2. Keep the tubes at 12-15°C when the reaction is finished. If the samples are not going to be processed at that moment, they can be stored at 4°C or at -20°C until use.

2.3. RECOMMENDED AMPLIFICATION CONTROLS

Some **positive clonal and polyclonal control DNAs** are supplied in the kit. It is recommended to amplify these DNAs with each batch of analyzed samples.

In order to control the potential presence of contamination from some samples to others, or from the handling of commonly used reagents during the handling of the samples, it is recommended to process a **blank sample without DNA**. This sample would carry the same reagents used in the DNA extraction from the samples and would follow a similar processing to the rest of samples, but, as it does not contain DNA, the result after the amplification should be absence of signal for all the samples.

Likewise, it is advisable to analyze every sample in duplicate, that is, from the same genomic DNA, **amplify in duplicate** for all the fragments. This way, the interpretation of doubtful results is made easier and it is verified with the duplicate the size of monoclonal peaks amongst a reactive polyclonal population.

3. ANALYSIS OF AMPLIFIED PRODUCTS

3.1. GEL ELECTROPHORESIS

Warning!: Given the high sensitivity of the amplification technique, which generates high quantities of a specific DNA fragment, this amplified product represents a significant source of contamination in the laboratory. It is recommended that handling and electrophoresis of the amplified products be carried out in a work area away from the sample processing area to avoid contamination of the samples with the amplified DNA, which could lead to false positive results.

The development of the amplified products can be carried out both in agarose gels (4%) and in polyacrylamide gels (6-8%) with TBE 0.5X buffer. The resolution in polyacrylamide gels is higher than that of agarose gels.

Vitro S.A. has kits for DNA electrophoresis in both agarose and polyacrylamide gels ready for use which also include all the reagents necessary for carrying out the electrophoresis: molecular-weight size marker, loading buffer, concentrated TBE buffer, EtBr. (**Cat. No.: MAD-003980M and MAD-003990M for agarose and polyacrylamide respectively**).

In order to differentiate products derived from mono- or polyclonal populations, it is recommended to carry out a "heteroduplex" analysis of the PCR products, by denaturing/renaturing the amplified products.

If clonal rearrangement of the TCRgamma gene has occurred, a "homoduplex" will be obtained, while in the case of polyclonal population, after the denaturation/renaturation process, "heteroduplex" with heterogeneous junctions will be formed.

3.1.1. PROCEDURE

1. Denature the PCR products at **94º C, 5 min**.
2. Re-nature by quickly transferring to ice (4 °C) and keep for 10-60 min.
3. Assemble the agarose or polyacrylamide gel in its appropriate cuvette and cover with electrophoresis 0.5X TBE buffer.
4. Take **20 µl of PCR product** and mix with **4 µl** of **6X loading buffer**.
5. Load the samples in the gel wells and place **10 µl** of **molecular weight-size marker** in one of the lanes.

6. Leave the electrophoresis work for **1-2 h. at 100 Volts**. The voltage and run time can be adapted depending on the type of gel, electrophoresis chamber, size of the amplified product, etc.

7. Stain with **0.5µg/ml EtB** in water or 0.5X TBE and visualize in transilluminator with UV light.

3.2. CAPILLARY ELECTROPHORESIS

All PCR mixes contain 6-FAM fluorochrome labeled primers, allowing these PCR products to be analyzed by capillary electrophoresis in addition to conventional electrophoresis (compatible with *ABI PRISM®* AB 310, 3100, 3130, 3130xl, *3500* and *3500xL* Genetic Analyzers).

Automated sequencers based on capillary electrophoresis, such as the ABI PRISM® 3100 or 3500 Genetic Analyzers by Applied Biosystems, are systems with a high rate of reproducibility and sensitivity in the sequencing and analysis of genomic fragments. The efficiency exceeds most sequence analyses based on the use of acrylamide gels. In addition, these systems allow the analysis of the results in a quick and precise way by means of specific software.

3.2.1. PREPARATION OF THE PCR PRODUCT

1. Mix in an Eppendorf tube of 0.2 ml: 15 µl of deionized formamide + 0.5 µl of the molecular weight standard + 1 or 2 µl of amplified DNA (this volume of amplified product can be modified if the fluorescent signal is outside the optimal range).

2. Homogenize in vortex and give a pulse.

3. Denature at **95°C x 5 min**, cool at 4°C and load in sequencer.

Electrophoresis conditions (3500 GENETIC ANALYZER):

- Polymer: POP-7 POLYMER
- Buffer: ANODE and CATHODE BUFFER CONTAINER 3500 SERIES
- Capillary: CAPILLARY ARRAY 8-CAP 50 CM
- Label: GeneScan 600 LIZ Size Standard
- Formamide: HI-DI FORMAMIDE
- Electrophoresis:
    - Oven Temperature (°C): 60
    - Run Voltage (KVolts): 19.5

    - PreRun Voltage (KVolts): 15
    - Injection Voltage (KVolts): 1.6
    - Run time (sec): 1330
    - Pre Run time (sec): 180 sec
    - Injection Time (sec): 8
    - Data Delay (sec): 1

Fluorochromes and filters:

Dye set	Filter set	Blue	Green	Yellow	Red	Orange
DS-31	D	6- FAM™	VIC®	NED™	ROX™	
DS-33	G5	6- FAM™	VIC®	NED™	PET™	LIZ®

3.2.2. OPTIMAL CONDITIONS FOR SAMPLE LOADING

In order to make sure that the DNA sample has been processed correctly and that there is sufficient DNA in quantity and quality to generate a valid result in the electrophoresis analysis, it is recommended that an aliquot of the PCR products (including IC) is loaded into a conventional EtBr-stained electrophoresis, prior to analysis by capillary electrophoresis.

If an excessive amount of PCR product is loaded into the capillary electrophoresis, the peaks in both the molecular weight standard and the product may be artefacted, resulting in false readings. Thus, an excess of one clonal sample can result in a spike pattern that would simulate what a polyclonal sample might be.

To avoid this problem, make sure that the intensity of the fluorescent signal is maintained between 400-31000 fluorescent units in the mode3500.

4. INTERPRETATION OF RESULTS

4.1. AMPLIFICATION INTERNAL CONTROL (IC)

An intense band or peak of **269 base pairs**, (capillary electrophoresis) should appear in all samples, indicating that the sample handling process and the quality of the DNA have been adequate. This amplification control is essential, especially in those samples obtained from paraffin-embedded material, where the quality and quantity of DNA obtained is unknown.

If no amplification band/peak appears with the IC, a negative amplification result of the TCR_{γ} fragments cannot be evaluated. This may be due to insufficient DNA obtained in the extraction process, to partially degraded DNA or the presence of PCR inhibitors in the sample. In the first two cases, DNA performance and quality can be improved by increasing the starting material or prolonging the incubation of the tissue with the lysis+protease buffer for another 24-48 hours.
If it is suspected that the sample may contain PCR inhibitors (ratios outside the expected range after measuring the concentration of purified DNA in a spectrophotometer), evaluable results can sometimes be obtained by diluting the test sample in order to reduce the presence of such inhibitors.

If after repeating the process no amplification is achieved, the sample should be reported as: "non-assessable for analysis of TCR_{γ} gene fragments by PCR".

4.2. REARRANGEMENT OF TCRgamma FRAGMENTS

The following table indicates the expected size range of the amplified fragments for each of the amplification mixes:

Table 2.

Primer mix	*Size Range*	*Clonal DNA positive control*	*Color (PCR tube)*
VJ-A	145-175 bp 175-195 bp 195-230 bp 230-255 bp	208 bp	Blue
VJ-B	80-110 bp 110-140 bp 160-195 bp 195-220 bp	113 bp	Purple

(1) The fragment size ranges indicated in Table 2 have been obtained with a 3500 Genetic Analyzer and analyzed with Genemapper. This size can vary between 1-6 bp depending on both the capillary electrophoresis equipment and the analysis software used. Once you have determined the size of the amplicon on your own

platform, you can consider this value as a reference and it will be reproducible in the rest of your electrophoresis.

(2) *The expected size ranges are estimated as a function of the positions of the primers and the expected insertion of nucleotides at the binding sites. However, they constitute a range of approximately 5-95% and true rearrangements with lower/higher sizes can occur as long as they are confirmed in the duplicates and in the absence of non-specific products in other samples.*

4.2.1 POSITIVE AND NEGATIVE AMPLIFICATION CONTROLS:

The **negative amplification control** performed with a **"blank" sample** should give no amplification in all the mixtures tested (including IC). If an amplified product is detected in any PCR mixture, it would indicate the presence of reagent contamination and the test would have to be repeated to validate it.
The **polyclonal positive control** should give multiple bands (gel) or peaks (capillary electrophoresis) within the size range indicated in Table 2 for each amplification mix.

The **positive clonal control** will give one single band or peak for each of the PCR mixes tested, the size of which is indicated in Table 2.

Any different than expected results obtained after analysis of the positive control DNA would indicate that the test is invalid and the problem samples could not be interpreted.

4.2.2 ANALYSIS OF THE TEST SAMPLE:

Before interpreting the results obtained with the test sample, it is necessary that all the controls included in the test, both positive and negative as well as the internal amplification control have been correct.

It is also recommended to follow the indications of the latest guide updates such as those of the EuroClonality/BIOMED-2 group for the interpretation of the results.

AGAROSE-POLYACRYLAMIDE GELS

The clonal rearrangement of the TCRgamma gene is visualized by electrophoresis through the presence of a single intense and sharp band within the expected size range (Figure 1). **A sample is considered to be "clonal" when a band with one or some of the tested amplification mixes is detected: VJ-A and VJ-B.**

If there is **no clonal rearrangement** and the test sample is composed of a polyclonal heterogeneous population of T cells, a heteroduplex will form and the result after electrophoresis will be a **multitude of bands within the size range, which are visualized in the gel as a wide and diffuse smear (Figure 1).**

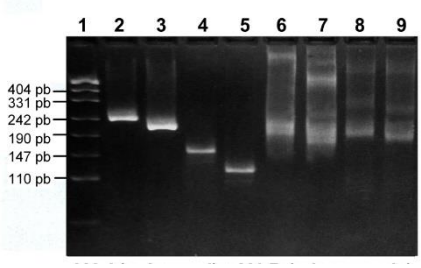

Figure 1. Analysis of results in 4% agarose gel. 1. MW standard, **2**. VJ-A mix, T monoclonal sample; **3.** VJ-A mix, T Cell-line; **4.** VJ-B mix, T monoclonal sample; **5.** VJ-B mix, T Cell-line; **6.** VJ-A mix, T polyclonal sample; **7.** Mix VJ-A, tonsil; **8.** VJ-B mix, T polyclonal sample; **9.** VJ-B mix, tonsil.

CAPILLARY ELECTROPHORESIS

The fluorochrome labeled amplification products are separated by capillary electrophoresis according to their size and are automatically detected by a laser.

• **Polyclonal lymphoproliferative process (Figure 2)**: Gaussian distribution of multiple amplified peaks of different size (within the expected size range) for each of the regions analyzed (VJ-A and VJ-B) of the TCR_{γ} gene representing a heterogeneous polyclonal population of T cells

It will be reported as "undetected clonality (detected polyclonality) for the TCR_{γ} gene in the analyzed sample under the conditions of this test".

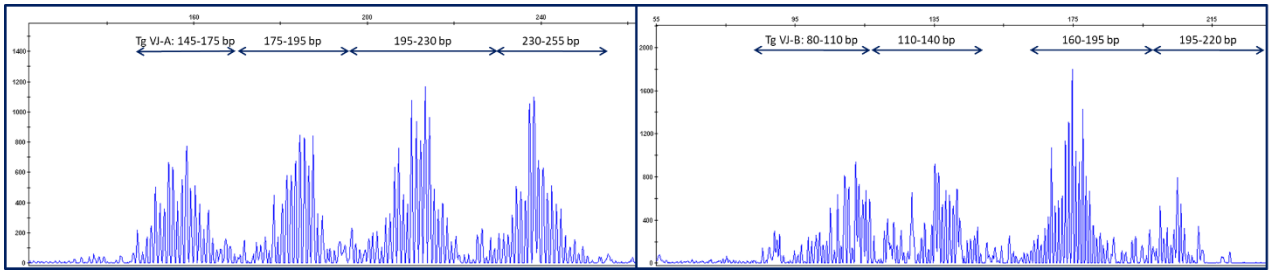

Figure 2: Electropherograms obtained after analysis by capillary electrophoresis of PCR-amplified products from the VJ-A and VJ-B regions of the TCR_{γ} gene (from left to right). They represent the typical Gaussian distribution corresponding to a polyclonal population with multiple amplified products of different sizes for each of the amplified regions

• **Clonal lymphoproliferative process (Figures 3 and 4):** one or two single and prominent peaks appear within the expected size range with all or some of the primer mixes tested (VJ-A and VJ-B) for the TCR_{γ} gene. These peaks are reproduced with the same size in the amplification duplicate and correspond to a clonal population (one peak) with a single amplified clone or biallelic/biclonal (two peaks) with two amplified clones (biclonal) within the size range analyzed.

Sometimes multiple peaks of different sizes may appear on the same electropherogram, some of which stand out in height from the others. The criterion for considering one or two clonal peaks is that they are at least 2.5 times higher than the height of the other adjacent peaks representing the polyclonal background and that the size of these peaks is confirmed in the PCR duplicate.

When the size difference between two dominant amplified peaks in the same region is ≤ 2 pb these peaks will be considered as a single peak especially if these peaks are the same height. This may be due to the fact that during PCR the polymerase could add an extra adenine not present in the target sequence at the end of the amplified fragment. If this does not occur equally in all the amplified fragments there will be a variation in +/- 1pb for the same original rearranged fragment.

These results would be reported as: "clonality detected for the TCR_{γ} gene in the sample analyzed under the conditions of this test"

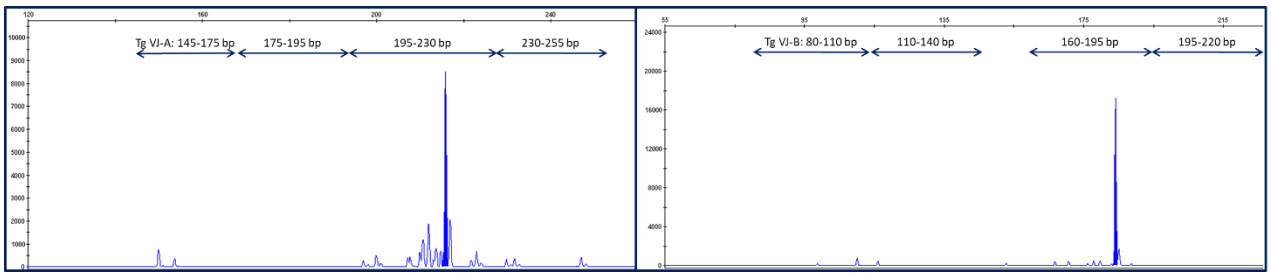

Figure 3: Electropherograms obtained after analysis by capillary electrophoresis of PCR-amplified products from the VJ-A and VJ-B regions of the TCR_{γ} gene (from left to right). They represent a single peak within the established size range (VJA: 195-230 pb and VJB: 160-195 pb) obtained from each of the primer mixes VJ-A or VJ-B corresponding to a clonal population with a single amplified clone.

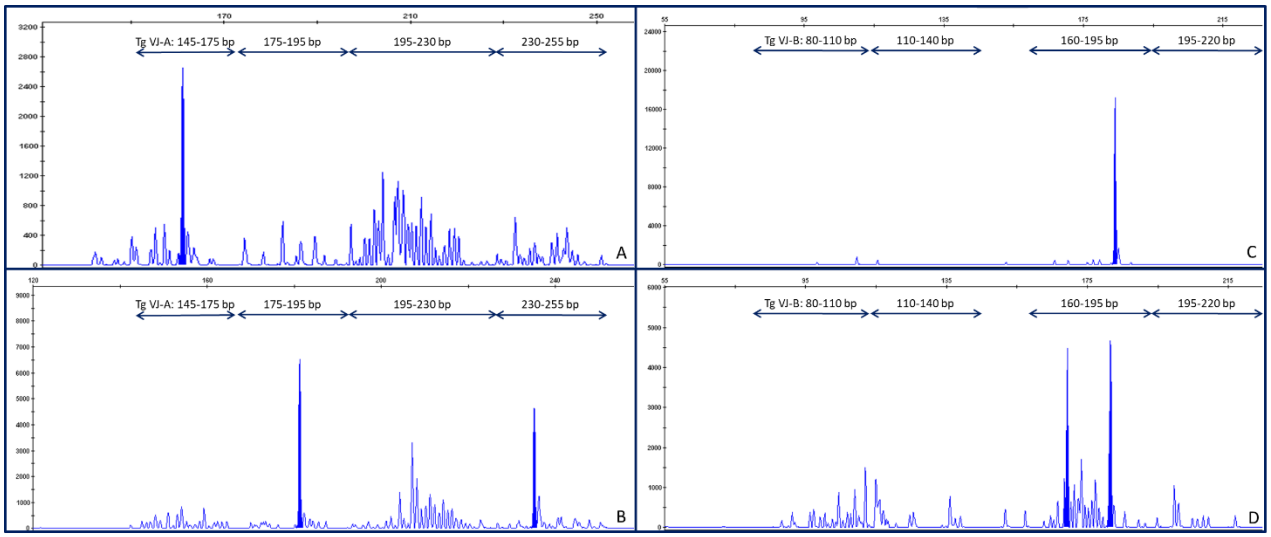

Figure 4: Electropherograms obtained after analysis by capillary electrophoresis of PCR-amplified products from the VJ-A and VJ-B regions of the TCR$_\gamma$ gene (from left to right). They represent different clonality patterns obtained from different samples. **A:** representation of a clonal pattern of a prominent peak with a height 2.5 times higher than the rest of peaks in the region of 145-175pb present in the sample after the amplification of the VJ-A region of the TCR$_\gamma$ gene. **B:** representation of a local pattern (biclonal/biallelic) of two prominent peaks (one in the region of 175-195pb and another one in the region of 230-255pb) with a height 2.5 times higher than the rest of the peaks present in the sample obtained after the amplification of the VJ-A region of the TCR$_\gamma$ gene **C:** representation of a clonal patter of single peak obtained after the amplification of the VJ-B region of the TCR$_\gamma$ gene (160-195 pb) **D:** representation of a clonal pattern (biclonal/biallelic) of two prominent peaks with a height of 2.5 times higher than the rest of peaks present in the sample obtained after the amplification of the VJ-B region of the TCR$_\gamma$ gene (size range 160-195 pb) In a clonal sample, any of the patterns described in all or some of the regions analyzed may appear.

- **Pseudoclonal lymphoproliferative process (Figure 5)**: presence of 1 or 2 peak(s) or multiple peaks (n ≥3) not reproducible in the duplicate. This can occur when there is an amplification from a DNA sample containing a low number of T cells, an infiltrate or a small sample (e.g. skin).

These results would be reported as: "undetected clonality for the TCR$_\gamma$ gene with the conditions of this test that could be associated with a shortage of T-cellularity in the sample".

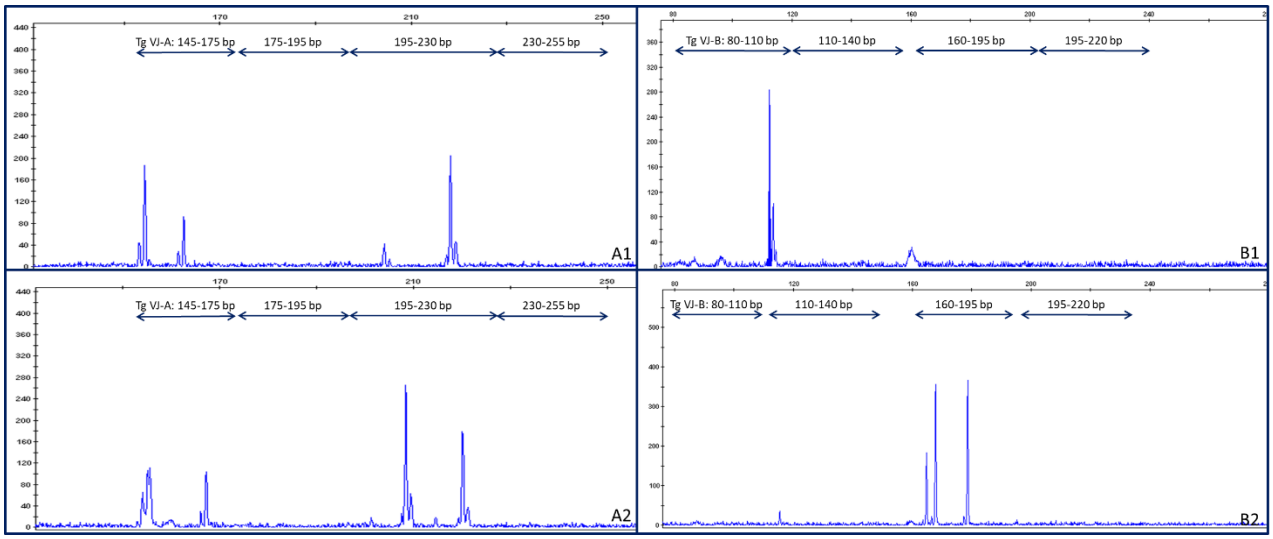

Figure 5: Electropherograms obtained after analysis by capillary electrophoresis of the PCR-amplified products from the VJ-A and VJ-B regions of the TCR$_\gamma$ gene (from left to right). They represent a pseudo-clonal amplification pattern with 1 or 2 prominent peaks that do not reproduce with the same size in the duplicate. **A1 and A2**: duplicates after amplification of the VJ-A region; **B1 and B2**: duplicates after amplification of the VJ-B region.

• **Oligoclonal lymphoproliferative process (Figure 6):** presence of multiple reproducible products (n≥ 3) in the duplicates as a consequence of the presence of an immune activation with dominant clones (e.g. infection, autoimmunity).

These results would be reported as: "oligoclonality/multiple clones detected for the TCR$_\gamma$ gene under the conditions of this test".

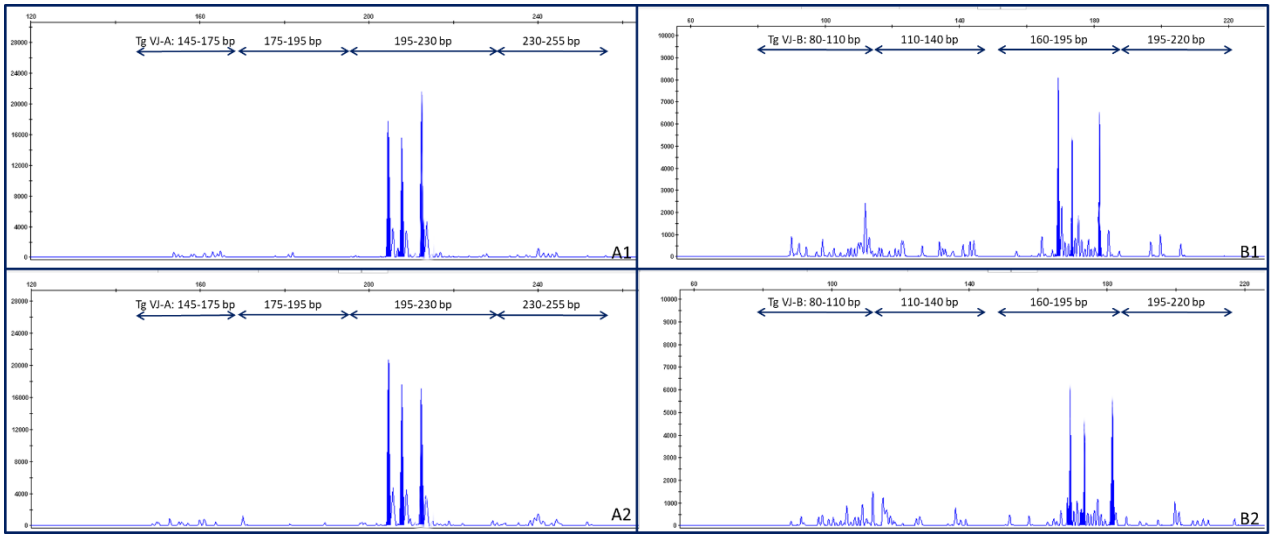

Figure 6: Electropherograms obtained after analysis by capillary electrophoresis of PCR-amplified products from the VJ-A and VJ-B regions of the TCR$_\gamma$ gene (from left to right). They represent an oligoclonal amplification pattern with three prominent peaks (in the region of 195-230 pb of the VJ-A mix and in the region of 160-195pb of the VJ-B mix) reproducing with the same size in the duplicate for each of the TCR$_\gamma$ gene regions analyzed. **A1 and A2**: representation of duplicates obtained after amplification of the VJ-A of the TCR$_\gamma$ gene **B1 and B2**: representation of duplicates obtained after amplification of the VJ-B region of the TCR$_\gamma$ gene.

5. LIMITATIONS OF THE TEST

In a sample containing a mix of tumor (clonal) T cells and an accompanying population of reactive T cells, the sharp band indicative of clonality may be masked by the rest of the multiple bands and not be visible by gel electrophoresis or capillary electrophoresis.

This method is capable of detecting between 2 and 5 tumor cells in 100 normal cells (Table 3). The results were obtained after testing all three amplification mixes with serial dilutions of a DNA sample from the cell line of T-cell lymphoma (JURKAT) with a DNA from peripheral blood lymphocytes.

Table 3. *Results of limit of detection*

Mix	***Limit of detection***
VJ-A	4 ng clonal DNA (2 % in total 0.2 µg)
VJ-B	10 ng clonal DNA (5 % in total 0.2 µg)

The TCRgamma gene is rearranged in more than 90% of T-cell acute lymphoblastic leukemias (T-ALL), T-cell prolymphocytic leukemias (T-PLL), T-cell large granular lymphocytic leukemias (T-LGL), and T-cell peripheral lymphomas (T-NHL), and in 75% of anaplastic large T-cell lymphomas. This gene also rearranges in about 60% of B-cell acute lymphoblastic leukemias (B-ALL), suggesting that this marker cannot be used for the establishment of B or T cell line in immature lymphoproliferative processes (Brüggeman et al, 2007).

A negative clonality result with this test does not exclude the diagnosis of lymphoma from other clinical, morphological, and immunophenotypic data.

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