TCR beta Rearrangements Molecular Analysis kit

Kit for the detection of rearrangements of the TCR beta gene

REF **Ref. MAD-003993TP-2** **20 determinations**

For in vitro diagnostic use only
Directive 98/79/EC

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1 INTENDED USE

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 B and T-cell lymphoproliferative processes.

TCR gene rearrangements play an important role in T lymphoid development. The TCR_{δ} locus is the first to be rearranged, the TCR_{γ} locus is re-arranged later, and if the rearrangements are functional they would lead to phenotypic expression of the $TCR_{\gamma\delta}$ receptor. Then, it rearranges the $TCR_{ß}$ with subsequent deletion of the TCR_{δ} and finally the TCR $_{\alpha}$ gene is rearranged. In the rearrangement of the $TCR_{ß}$ gene, the segments $D_{ß}$-$J_{ß}$ are first merged and then recombined with segment $V_{ß}$. Rearrangements of the TCR_{α} locus take place in the final stages of T lymphoid development and if functional rearrangements lead to expression of the TCR receptor$_{\alpha\beta}$. These $TCR_{\gamma\delta}$ or $TCR_{\alpha\beta}$ configurations appear in the post-thymic T cells. Most mature cell T lymphomas express $TCR_{\alpha\beta}$ combined with a biallelic deletion of TCR_{δ}, while only a minority express $TCR_{\gamma\delta}$.

The $TCR_{ß}$ gene is rearranged in all cells with TCR $_{\alpha\beta}$ phenotype, and also in many $TCR_{\gamma\delta}$. In theory, all lymphocytes with TCR $_{\alpha\beta}$ phenotype have rearranged TCR_{γ} genes, and most of the positive ones for $TCR_{\gamma\delta}$ phenotype have reordered the $TCR_{ß}$ genes. PCR analysis of $TCR_{ß}$ gene rearrangements represents a complementary study to be performed when there is reasonable suspicion of T-cell lymphoma with inconclusive PCR-TCR_{γ} results. The combination of both studies would allow the detection of T-cell clonality in a high percentage of T-cell lymphoma samples.

TCR beta Rearrangements Molecular Analysis kit is an *in vitro* diagnostic kit that allows the qualitative detection of the presence of clonality in lymphoproliferative processes of T origin.

The product is intended solely for professional use in a laboratory as a diagnostic aid in patients with suspected lymphoproliferative diseases, in cases where histomorphological and immunohistochemical data are not sufficient for differential diagnosis between a reactive process and a malignant lymphoma, and not as a drug, for home use or other purposes.

2 PRINCIPLE OF THE METHOD

This is a non-automated process in which, after DNA extraction from clinical samples of different origin (peripheral blood lymphocytes, fresh frozen tissue or formalin-fixed and paraffin-embedded tissue), amplification of the rearranged $V_{ß}J_{ß}$ and $D_{ß}J_{ß}$ segments of the $TCR_{ß}$ gene is carried out by PCR.

Given the diversity of sequences of these TCR genes, multiple targeted primers are used against conserved regions flanking V, D and J segments in order to detect as many clonal rearrangements as possible. 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 amplicons generated after the PCR can be detected by electrophoresis on agarose/polyacrylamide gels or capillary electrophoresis.

For the interpretation of the results, it is also recommended to follow the guidelines of the latest guide updates such as those of the EuroClonality/BIOMED-2. Likewise, the result obtained is a complementary datum that must be interpreted by a professional in the content of the clinical history of the patient, as well as other morphological and immunophenotypic data.

3 COMPONENTS

The TCR beta Rearrangements Molecular Analysis kit is marketed in a single format and has the following features:

- It contains two amplification mixes $V_ßJ_ß$-A, $V_ßJ_ß$-B to amplify the $V_ß$-$J_ß$ region, a $D_ßJ_ß$ mix (C) to amplify the $D_ß$-$J_ß$ region of the TCR beta gene and an internal amplification control (IC) to check the DNA quality.
- The amplification mixtures are presented in mono-test format in 0.2 ml PCR tube strips, identified in different colors.
- All PCR mixes include primers labeled in their 5' end with fluorochrome 6-FAM, allowing automatic analysis of fragments by capillary electrophoresis (compatible with Genetic Analyzers by ThermoFisher Scientific; series 310, 3100, 3500, 3730 and SeqStudio).
- 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 amplification DNA positive controls are included.

The components included in the kit are summarized in **table 1:**

Components of TCR beta Rearrangements Molecular Analysis Kit			
REAGENT	**REFERENCE**	**COMPONENTS**	**AMOUNT**
Amplification mixes (mono-test in tubes of 0.2)	MAD-003993TP-VJA-T	VßJß-A(6-FAM) (mix A of oligonucleotides from regions V and J of the TCRß gene, dNTP and buffer solution)	20 red-colored PCR tubes (46 µl)
	MAD-003993TP-VJB-T	VßJß-B (6-FAM) (mix B of oligonucleotides from regions V and J of the TCRß gene, dNTP and buffer solution)	20 green-colored PCR tubes (46 µl)
	MAD-003993TP-DJ-T	DßJß (6-FAM) (mix of oligonucleotides from regions D and J of the TCRß gene, dNTP and buffer solution)	20 violet-colored PCR tubes (46 µl)
	MAD-003993TP-P53-T	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-F122-2	Hot Start II DNA Polymerase	2 x 60 µl

DNA positive controls	MAD-003993T2	DNA clonal positive control T JURKAT (VßJß-A and DßJß)	1 x 50 µl
	MAD-003993T3	DNA clonal positive control T MOLT3 (VßJß-B)	1 x 20 µl
	MAD-003994B3	DNA polyclonal positive control	1 x 50µl

Table 1: Reagents supplied in the TCR beta Rearrangements Molecular Analysis kit

Limited Use Label License

The purchase price of this Product includes a limited, non-transferable license under U.S. and foreign patents owned by BIO-RAD Laboratories, Inc. to use this Product. No other license under these patents is conveyed expressly or by implication to the purchaser by the purchase of this Product.

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

4 ADDITIONAL REQUIRED MATERIAL NOT SUPPLIED

4.1 Reagents and materials

- Disposable gloves
- DNase/RNase-free filtering pipette tips.
- DNase/RNase-free Eppendorf tubes of 1.5/0.6/0.2 ml
- Cooling plate (4 °C).
- Xylene (optional)
- Ethanol 100%
- DNA extraction kit: reagents to perform genomic DNA extraction from blood samples, cell buttons, fresh tissues, or buffered formalin-fixed paraffin-embedded tissues. In the case of paraffin-embedded samples, the Vitro S.A. kit: PARAFFIN TISSUE PROCESSING KIT Ref: MAD-003952M includes all the reagents necessary to carry out the extraction.
- PBS buffer (for the extraction of lymphocytes from whole blood)
- TE buffer 1x (10 mM Tris, 1 mM EDTA pH 8.0)
- Reagents for electrophoresis of amplified DNA (agarose or polyacrylamide gels and auxiliary reagents for electrophoresis): 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).
- Capillary electrophoresis reagents recommended by the supplier for their automated sequencers; ThermoFisher Scientific Genetic Analyzers (series 310, 3100, 3500, 3730 and SeqStudio series).

4.2 Equipment

- Nucleic acids automated extraction system.
- Spectrophotometer
- Microcentrifuge.
- Automatic micropipettes: P1000, P200, P20 and P2.
- Thermostatic bath / heater.

- Thermal cycler.
- Power supply.
- DNA electrophoresis chamber.
- UV Transilluminator.
- Gel documentation system.
- Sequencer with capillary electrophoresis.

5 STORAGE AND STABILITY CONDITIONS

The TCR beta Rearrangement Molecular Analysis kit should be transported and stored between -10 and -30 °C. However, in addition to the recommended transport between -10 and -30 °C, transport at refrigerated temperature (2 °C - 8 °C) is also possible, provided that the transit time does not exceed a maximum of fifteen days. In any case, once the kit is received, it must be stored at a temperature between -10 and -30 °C.

The amplification mixes of the TCR beta Rearrangements Molecular Analysis kit are sensitive to physical state changes and have been shown to resist up to five freeze/thaw cycles. These mixes contain primers labeled with fluorescent molecules and should be stored away from direct light.

The DNA polymerase is sensitive to physical state changes and it must not undergo more than ten freeze-thaw cycles. It must be stored at a temperature between -10 and -30 °C.

The positive amplification controls are sensitive to physical state changes and it must not undergo more than ten freeze-thaw cycles. These controls should be stored at 2-8 °C after thawing. It is advisable to handle the positive control vials separately from the clinical samples to avoid potential contamination which might yield false positives.

If stored at recommended temperature, the PCR reagents are stable until the expiration date indicated. The PCR reagents must be stored in areas free of DNA or PCR products contamination.

6 WARNINGS AND PRECAUTIONS

- **Read the instructions for use before using this product**.
- The kit must be handled by qualified technicians in molecular biology techniques applied to diagnosis.
- Do not use any component of the kit after the expiration date.
- **Amplification mixes** should be thawed before use and handled on ice or on a cold plate protected from light.
- The **positive amplification controls** must be thawed at room temperature, mixed well and centrifuged briefly before use.
- The **safety and disposal precautions** are described in the Safety Data Sheet of this product. This 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 Safety Data Sheet of this product can be downloaded in the web page www.vitro.bio or requested at regulatory@vitro.bio.
- TCR beta Molecular Analysis kit uses as starting material genomic DNA previously extracted from

clinical samples of different origin. Different DNA extraction protocols that have been validated with this kit are listed in section 7. It is the client's responsibility to include the necessary controls to verify that the system of extraction of the used genetic material works properly.

- **General considerations to avoid the contamination with PCR product:** 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 greatest contamination source is normally the same amplified PCR product. Therefore, it is recommended to carry out the handling of the amplified products in a different area than the one the PCR reaction is performed. It is recommended to work on different pre- and post-PCR areas where the handling of the test DNA and preparation of the PCR tubes (pre-PCR) and the handling and electrophoresis of the amplified products (post-PCR) are performed. These areas must be physically separated and different laboratory material must be used (laboratory coats, pipettes, tips, etc.) to avoid the contamination of the samples with the amplified DNA, which could lead to false positive diagnoses. The workflow must always go in a single direction, from the pre-PCR area to the post-PCR area and never the opposite way. The material and personal flow from the post-PCR area to the pre-PCR area must be avoided. **It is recommended to include negative amplification controls** containing all the reagents handled in the kit, from the extraction to the amplification, except for the DNA sample, in order to detect and control any possible contamination of the reagents with test samples or amplified products. The electrophoresis of this control should be negative, absence of bands/peaks. In this way it is verified that there is no contamination of patient DNA and/or amplified DNA in the pre-PCR area.
- **Waste disposal:** The handling of wastes generated by the use of the products commercialized by VITRO S.A. must be performed according to the applicable law in the country in which these products are being used. As reference, the following table indicates the classification of wastes generated by this kit according to the European Law, specifically according to the European Commission Decision of December 18, 2014 amending decision 2000/532/CE on the list of waste pursuant to Directive 2008/98/EC of the European Parliament and of the Council:

POTENTIAL WASTE GENERATED AFTER USING THIS PRODUCT	ELW CODE*	TYPE OF WASTE ACCORDING TO ELW*
1. Liquid waste disposal	161001	"Aqueous liquid wastes containing dangerous substances" after adding 10% of the total volume of a disinfectant agent. If the disinfection is not carried out, this waste must be considered as "waste whose storage and disposal is subjected to special requirements in order to prevent infection"
2. Consumables (tubes, tips, etc.) 3. Any element that has been in contact with the starting genetic material	180103	"Waste whose collection and disposal is subject to special requirements in order to prevent infection"
4. Container for reagents used classified as dangerous (according to the Safety Data Sheet)	150110	"Containers containing waste or contaminated by dangerous substances"

Table 2. Classification of wastes generated by this kit according to the European Legislation. *ELW: *European Legislation of Waste.*

*Note: This classification is included as a general guideline of action, being under the final responsibility of the user the accomplishment of all the local, regional and national regulations on the disposal of this type of materials.

7 GENOMIC DNA EXTRACTION FROM DIFFERENT CLINICAL SAMPLES

TCR beta Rearrangements Molecular Analysis kit may be used with different types of samples: peripheral blood lymphocytes, fresh, frozen or buffered formalin-fixed and paraffin-embedded tissue. Depending on the starting clinical sample, a specific genomic DNA extraction protocol will be used. In addition, manual or automatic protocols can be used for which different commercially available extraction equipment is used. The list of platforms with which the kit has been validated is shown in Table 3. The use of any other extraction system has not been validated and it must be previously verified by the user.

Equipment	Kit
Maxwell® 16 System (PROMEGA)	Maxwell® 16 FFPE Tissue LEV DNA Purified Kit (PROMEGA)
	Maxwell® 16 LEV Blood DNA kit (PROMEGA)
Nextractor NX-48S (Genolution)	FFPE DNA kit (Genolution)

Table 3: Automated genomic DNA extraction platforms and validated protocols with TCR beta Rearrangements Molecular Analysis Kit

Manual genomic DNA extraction protocols for each type of clinical sample validated with this kit are described below:

7.1 DNA extraction from paraffin-embedded tissue

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 should be stored at 15-25 °C and the remaining components should be kept at -10 and -30 °C.

1. Take 1-4 tissue sections (depending on the amount of material present in each section) 10 µm thick and place in a 1.5 ml microcentrifuge tube using a needle or tweezers.

2. Add **500 µl** of **mineral oil** (included in the kit) and heat on heating block 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 heating block.

8. Heat at **95º C** on heating block 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.

7.2 DNA extraction from 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 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.*

7.3 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 3000- 3400 rpm).

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

3. Wash with **5 ml** of **TE 1X** buffer 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 **TE 1X** buffer 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.*

8 AMPLIFICATION REACTION

8.1 Amplification reaction of the DNA from the test sample

For each sample of test DNA to be analyzed, 4 mixes will be amplified: $V_{ß}J_{ß}$-A, $V_{ß}J_{ß}$-B y $D_{ß}J_{ß}$ of the $TCR_{ß}$ gene and mix of internal control (IC). It is recommended to preserve the mixes of the light, since they contain primers labeled with fluorescence.

Procedure:

1. Thaw one red tube (VßJß-A), one green tube (VßJß-B), one purple tube (DßJß-C) and one yellow tube (IC) for each sample (tube and cap strips are divisible by cut separation). The rest of the tube strip with PCR mix that will not be used at the time should remain stored at -10 to -30 °C in the original package.
2. Centrifuge briefly and keep in ice.
3. Add to each of them following this order:
   - **1 µl** of the enzyme Hot Start II DNA Polymerase
   - **3 µl** of the **DNA sample***

   *If DNA of known concentration is available, it is recommended to add between 50-200ng of DNA.
4. Homogenize the mix by pipetting and centrifuge for 5 seconds in microcentrifuge to remove bubbles.
   Note: *It is important to keep the tubes in a cold plate (4ºC) up to the moment of placing them in the thermal cycler in order to avoid non-specific binding of primers.*
5. Place the tubes in the thermal cycler and amplify according to the amplification program below:

PCR PROGRAM		
98°C	2 min	1 cycle
98°C	10 sec	35 cycles
60°C	10 sec	
72°C	15 sec	
72°C	1 min	1 cycle
8°C	∞	

Table 4: PCR program.

The following thermal cyclers have been validated with the TCR beta Rearrangements Molecular Analysis kit:

- Veriti (Applied Biosystems)
- MJMiniCycler (BioRad)
- My-Cycler (BioRad)

- TC3000 speed Dial (Techne)
- Tadvanced (Biometra)

6. Keep the tubes at 8-10 °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.

8.2 Recommended amplification controls

Some **positive clonal and polyclonal control DNAs (positive amplification controls)** 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 (negative amplification control)**. 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 each 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.

9 ANALYSIS OF AMPLIFIED PRODUCTS

9.1 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/re-naturing the amplified products.

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

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.

9.2 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 automatic sequencers, such as the Genetic Analyzers by ThermoFisher Scientific (series 310, 3100, 3500, 3730 and SequStudio).

Automatic sequencers based on capillary electrophoresis 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 fast and precise way by means of specific software. Follow the manufacturer's instructions for the installation, use and maintenance of this systems.

To adjust the conditions for capillary electrophoresis follow the manufacturer's instructions and guidelines ("DNA Fragment Analysis by Capillary Electrophoresis guide" by ThermoFisher) depending on the type of capillary electrophoresis system and the type of capillary/polymer used. It is recommended to use the PCR products obtained from the positive amplification controls for the optimization of the capillary electrophoresis conditions, once the presence of bands for each of them has been verified in conventional electrophoresis according to the values indicated in table 7. Once determined in the platform, you will be able to consider this configuration as a reference and it will be reproducible in the rest of your electrophoresis.
To carry out the spectral calibration of the Genetic Analyzers (in the SeqStudio systems, this calibration is automatic), the "standard matrix" must be selected. This calibration prepares the system for the detection of the fluorochromes with which the primers of the amplification mixtures (6-FAM) are labeled. The choice of the standard matrix depends on the combination of fluorochromes ("dye set") to be detected and the Genetic Analyzer available. In this case two fluorochromes are used, 6-FAM for the primers and the

fluorochrome with which the molecular weight marker ("size standard") is labeled. Molecular weight markers use ROX, LIZ or TAMRA fluorochromes and are of the user's choice.

Matrix Standard	DS-30	DS-31	DS-33	DS-34
Dye set	D	D	G5	C
	Fluorochromes			
Blue	6- FAM™	6- FAM™	6- FAM™	6- FAM™
Green (not applicable)	HEX™	VIC™	VIC™	TET™
Yellow (not applicable)	NED™	NED™	NED™	HEX™
Red	ROX™	ROX™	PET™	TAMRA™
Orange			LIZ®	
	Genetic Analyzer			
3500	yes	yes	yes	no
3730/3730xl	yes	yes	yes	no
3130/3130xl	yes	yes	yes	no
310	yes	yes	yes	yes

Table 5: "Matrix standard" and "Dye set" for each Genetic Analyzer and correspondence with the combination of fluorochromes used in the primers (6-FAM) and the molecular weight marker (ROX, LIZ or TAMRA).

The following table shows the recommended fluorescence intensity range in relative fluorescence units (RFU) for optimal results ("DNA Fragment Analysis by Capillary Electrophoresis guide" by ThermoFisher):

Genetic Analyzer	Recommended fluorescence intensity range (RFU)	Fluorescence saturation (RFU)
Series 3500	175-10000	30000
Series 3730	150-10000	30000
Series 3130	150-4000	8000
310	150-4000	8000

Table 6: Adequate intensity ranges for each of the Genetic Analyzers

The protocol used for 3500 Genetic Analyzer is described below. This indications are illustrative and it is recommended to optimize them in the system used.

Procedure for the preparation of the PCR product:

1. Prepare a stock mix with 9 µl deionized formamide + 0.5 µl of the molecular weight standard for each PCR product to be loaded into the capillary electrophoresis.
2. Homogenize in vortex and centrifuge briefly.
3. Distribute 9.5 µl of the above mixture for each PCR product to be analyzed on a PCR plate.
4. Add 1 µl of PCR product (this volume of amplified product can be modified if the fluorescent signal is outside the optimal range) to each of the above mixtures.
5. Denature at **95 °C x 5 min**, cool to 4°C and centrifuge briefly.
6. The plate is ready to be loaded into the sequencer according to the instrument instructions.

Conditions of electrophoresis:

- 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

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, an aliquot of the PCR products (including IC) can be 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, artifactual peaks may be obtained. Thus, an excess of a clonal sample may result in a spike pattern that would simulate what could be a polyclonal sample, in such cases it is recommended to dilute the PCR product before loading into capillary electrophoresis.

10 QUALITY CONTROL PROCEDURE

The TCR beta Rearrangements Molecular Analysis kit consists of several controls to evaluate the quality of the results.

- **Amplification internal control (IC):** An intense band or peak of 269 base pairs (pb), (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 appears with the IC, a negative amplification result of TCR beta 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 and 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 TCR beta gene fragment analysis by PCR".

- **Polyclonal positive amplification control**: should give multiple bands (gel) or peaks (capillary electrophoresis) within the size range indicated in Table 7 for each amplification mix.

- **Positive clonal amplification control:** will give one single band or peak for each of the PCR mixes tested, the size of which is indicated in Table 7.

- **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.

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.

Table 7 shows the expected size range of the amplified fragments for each of the amplification mixtures, as well as the expected patterns for the amplification controls:

TCR beta Rearrangements Molecular Analysis kit				
Amplification mix	**Expected sizes range (bp)**	**Expected pattern for each amplification control**		
		Clonal positive control: • **JURKAT for $V_ßJ_ß$-A y $D_ßJ_ß$** • **MOLT for $V_ßJ_ß$-B**	**Polyclonal positive control**	**Negative control**
$V_ßJ_ß$-A (Red tube)	240-280	Band/Peak of 265 pb	Multiple peaks/bands between 240-280 pb	No bands/peaks
$V_ßJ_ß$-B (Green tube)	240-285	Band/Peak of 274/263 pb	Multiple peaks/bands between 240-285 pb	No bands/peaks

$D_ßJ_ß$ (Purple tube)	170-210 and 285-325	Band/Peak of 309 pb	Multiple bands between 170-210 / 285-325 pb	No bands/peaks
CI (Yellow tube)	269	Band/Peak of 269 bp	Peak of 269 bp	No band/peak

Table 7: Expected size range and pattern for amplification controls with each PCR mix.

(1) The fragment size ranges indicated in Table 7 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/upper sizes can occur as long as they are confirmed in the duplicates and in the absence of non-specific products in other samples.

11 INTERPRETATION OF RESULTS

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

It is 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**.** Likewise, the result obtained is a complementary datum that must be interpreted by a professional in the content of the clinical history of the patient, as well as other morphological and immunophenotypic data.

11.1 Agarose/polyacrylamide gels

- **Clonal lymphoproliferative process (Figure 1):** Clonal rearrangement of the TCR beta gene is visualized by electrophoresis by the presence of a single sharp, intense band within the expected size range. A sample is considered to be "clonal" when a band is detected with all or any of the two amplification mixes tested; VßJß-A, VßJß-B and/or DßJß (Figure 1).
  These results would be reported as: "clonality detected in the sample analyzed for the TCR beta gene with the conditions of this test".
- **Polyclonal lymphoproliferative process (Figure 1):** If there is no clonal rearrangement and the test sample is composed of a heterogeneous polyclonal population of T cells, a heteroduplex will form and the result after electrophoresis will be a multitude of bands in all amplification mixes within the expected size range, which are visualized on the gel as a broad, diffuse smear.
  It will be reported as "clonality not detected (polyclonality) for the TCR beta gene in the sample tested under the conditions of this test".
- **Non-assessable:** No band in all the amplification mixes. No specific product possibly due to sample shortage/absence of lymphocytes/insufficient DNA quality or quantity. Absence of TCR beta rearrangement.

It will be reported as "non-valuable sample for TCR beta gene fragment analysis by PCR".

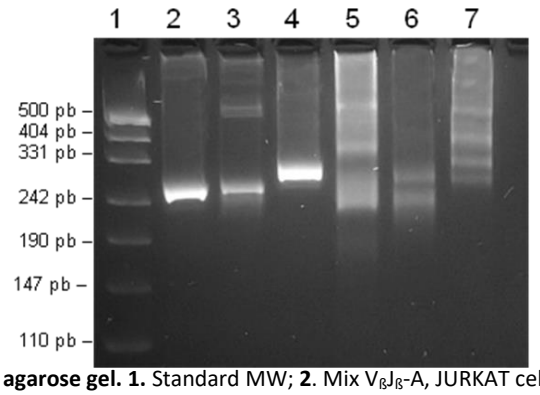

Figure 1. Analysis of results in 4% agarose gel. 1. Standard MW; **2**. Mix VßJß-A, JURKAT cell-line; **3.** Mix VßJß-B, MOLT 3; **4.** Mix DßJß, JURKAT cell-line; **5.** Mix VßJß-A, tonsil; **6.** Mix VßJß-B, tonsil; **7.** Mix DßJß, tonsil.

11.2 Capillary electrophoresis

The 6-FAM 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 (VßJß-A, VßJß-B and DßJß) of the TCR beta gene representing a heterogeneous polyclonal population of T cells

It will be reported as “undetected clonality (detected polyclonality) for the TCR beta gene in the analysed sample under the conditions of this test”.

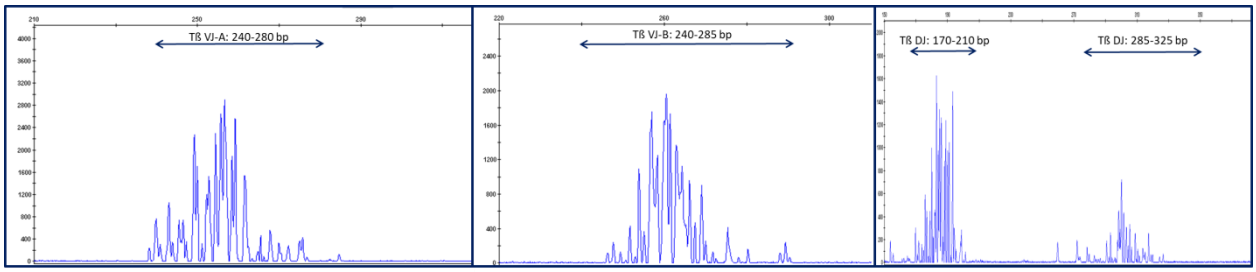

Figure 2: Electropherograms obtained after analysis, by capillary electrophoresis, of the PCR-amplified products of the VßJß-A, VßJß-B and DßJß regions of the TCRß gene (from left to right). They represent the typical Gaussian distribution corresponding to a polyclonal population with multiple amplified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

- **Clonal lymphoproliferative process (Figures 3 and 4):** one or two single or prominent peaks appear within the size ranges analyzed, in at least one of the primer mixes tested (VßJß-A, VßJß-B and DßJß) for the TCRß 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 (Figure 4 A)

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 TCRß gene in the sample analyzed under the conditions of this test"

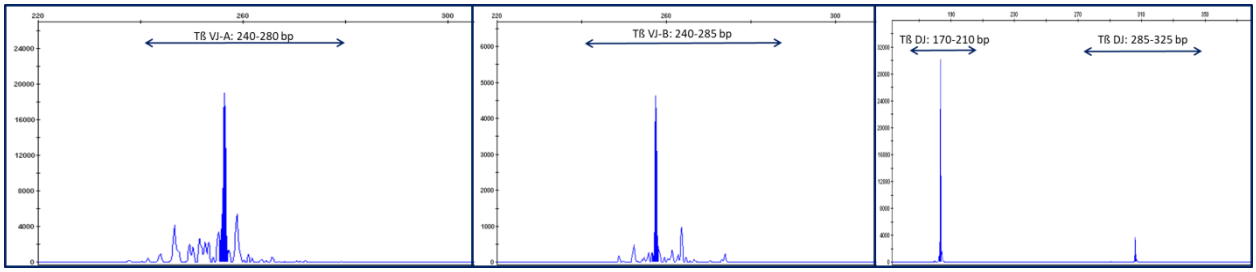

Figure 3: Electropherograms obtained after capillary electrophoresis analysis of the PCR-amplified products of the VßJß-A, VßJß-B and DßJß regions of the TCRß gene (from left to right)**.** They represent a single or prominent peak within the established size range obtained with each of the VßJß-A, VßJß-B or DßJß primer mixes of the TCRß gene and corresponding to a clonal population with a single amplified clone.

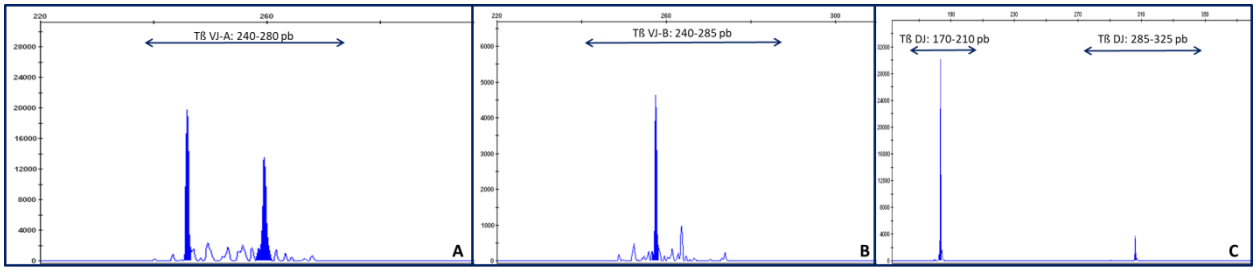

Figure 4: Electropherograms obtained after analysis, by capillary electrophoresis, of PCR-amplified products from the VßJß-A, VßJß-B or DßJß regions of the TCRß gene (from left to right). They represent different clonality patterns obtained from different samples. **A:** representation of a clonal pattern of two prominent peaks with a height 2.5 times higher than the rest of the peaks present in the sample obtained after amplification of the VßJß-A region of the TCRß gene. **B:** representation of a prominent clonal peak pattern with a height 2.5 times higher than the rest of the peaks present in the sample after amplification of the VßJß-B region of the TCRß gene. **C**: representation of a clonal pattern of single peaks obtained after amplification of the DßJß region of the TCRß gene In a clonal sample any of the described patterns can appear in all or some of the analyzed regions.

• **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 TCRß gene with the conditions of this test that could be associated with a shortage of T-cellularity in the sample”.

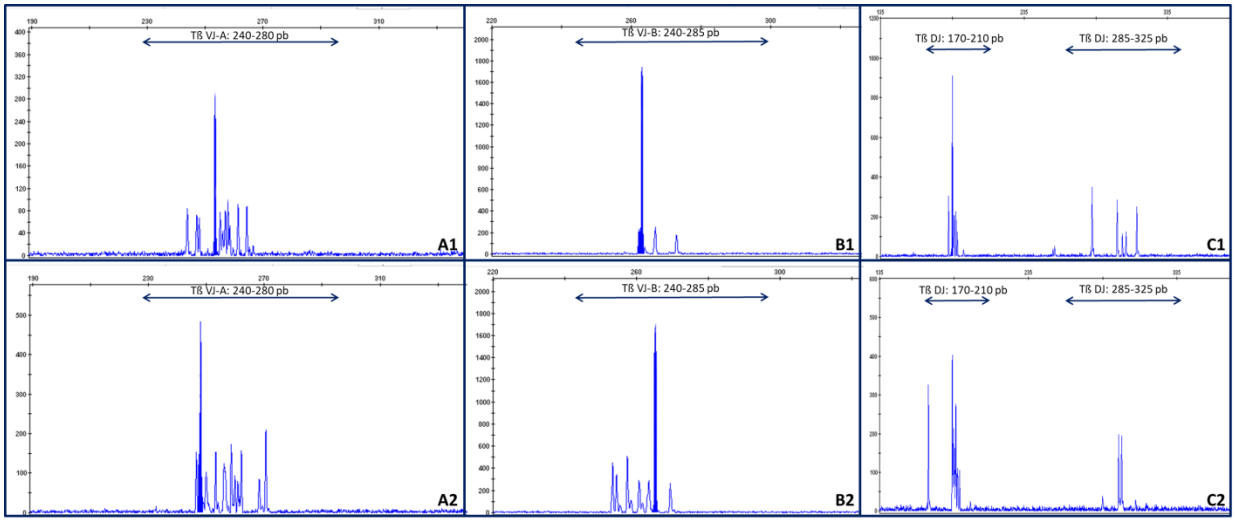

Figure 5: Electropherograms obtained after analysis, by capillary electrophoresis, of the PCR-amplified products of the $V_ßJ_ß$-A, $V_ßJ_ß$-B or $D_ßJ_ß$ regions of the $TCR_ß$ gene (from left to right). They represent a pseudo-clonal amplification pattern with 1 or 2 prominent peaks that do not reproduce with the same size in the duplicate. **A1 and A2**: duplicates after amplification of the $V_ßJ_ß$-A region. **B1 and B2**: duplicates after amplification of the $V_ßJ_ß$-B region. **C1 and C2**: duplicates after amplification of the $D_ßJ_ß$ region.

- **Oligoclonal lymphoproliferative process (Figure 6):** presence of multiple reproducible products (n≥ 3) in the duplicate 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 $TCR_ß$ gene under the conditions of this test".

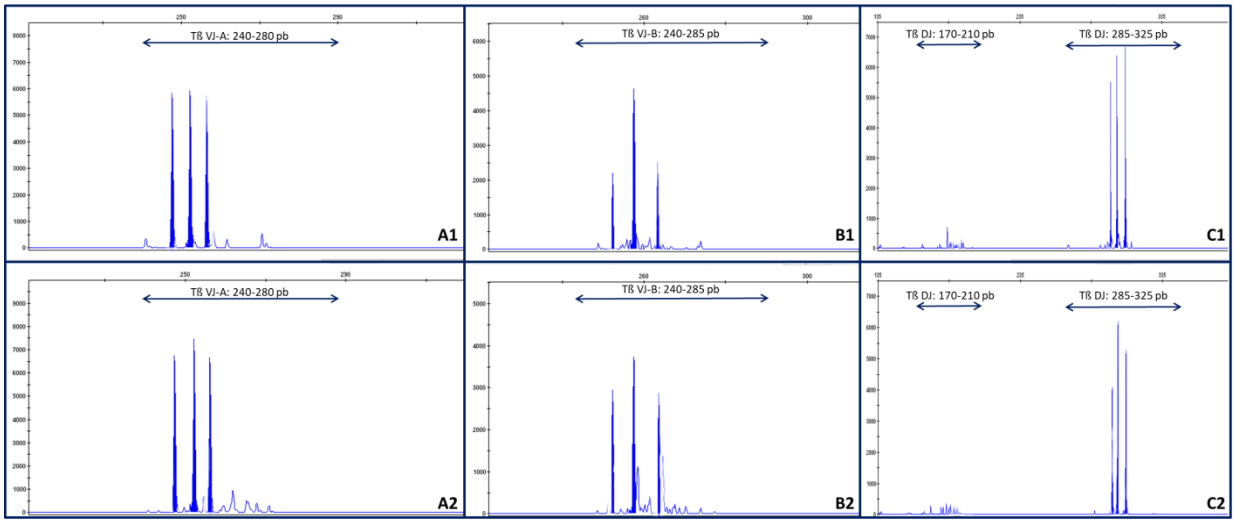

Figure 6: Electropherograms obtained after analysis, by capillary electrophoresis, of the PCR-amplified products of the $V_ßJ_ß$-A, $V_ßJ_ß$-B or $D_ßJ_ß$ regions of the $TCR_ß$ gene (from left to right). They represent an oligoclonal amplification pattern with three prominent peaks reproducing with the same size in duplicate for each of the $TCR_ß$ gene regions analyzed. **A1 and A2**: representation of duplicates obtained after amplification of the $V_ßJ_ß$-A region of the $TCR_ß$ gene **B1 and B2**: representation of duplicates obtained after amplification of the $V_ßJ_ß$-B region of the $TCR_ß$ gene. **C1 and C2**: duplicates after amplification of the $D_ßJ_ß$ region of the $TCR_ß$ gene.

- **Non-assessable:** No peaks in all the amplification mixes. No specific product possibly due to sample shortage/absence of lymphocytes/insufficient DNA quality or quantity. Absence of TCR beta rearrangement.

  It will be reported as "non-valuable sample for TCR beta gene fragment analysis by PCR".

12 PERFORMANCE CHARACTERISTICS

12.1 Analytical functioning

12.1.1 *Repeatability*

The repeatability of the method was analyzed with samples containing a total of 150ng of DNA with different percentages of DNA from a T lymphoma cell line in a background of peripheral blood lymphocyte DNA. Two different percentages of the T lymphoma cell line were used and four replicas of each were made. The test was performed by the same operator, in a single location and using the same reagent lot. All results were analyzed by capillary electrophoresis with the Genemapper software. The interpretation of the results was carried out taking into account the indications of the latest updates of the EuroClonality/BIOMED-2 group guide for the interpretation of the results.

Fragment	% clonal DNA	Positive/tested	% positive
VßJß-A	20	4/4	100%
	10	4/4	100%
VßJß-B	30	4/4	100%
	20	4/4	100%
DßJß	30	4/4	100%
	20	4/4	100%
Internal Control	10	4/4	100%
	5	4/4	100%

Table 8: Repeatability test for each amplification fragment of the TCRbeta gene and internal control included in the TCRbeta Rearrangements Molecular Analysis Kit.

12.1.2 *Reproducibility*

The reproducibility of the test was proven by processing samples containing 150ng of DNA with different percentages of DNA from the T lymphoma cell line contained in a background of peripheral blood lymphocyte DNA. Each sample was processed 4 times in different days under two different conditions: two lots of reagents and different equipment and operator. No false positive results were obtained (100% of the polyclonal samples gave negative results). Clonality percentages are shown in the table below:

% clonal DNA	Fragment	Condition	Clonality/Total	% Clonality
30%	VβJβ-B	1	4/4	100
		2	4/4	100
	DβJβ	1	4/4	100
		2	4/4	100
20%	VßJß-A	1	4/4	100
		2	4/4	100
	VβJβ-B	1	4/4	100
		2	4/4	100
	DβJβ	1	4/4	100
		2	4/4	100

10%	Vβ-Jβ-A	1	4/4	100
		2	4/4	100
	Vβ-Jβ-B	1	2/4	50
		2	3/4	75
	Dβ-Jβ	1	2/4	50
		2	2/4	50
5%	Vβ-Jβ-A	1	3/4	75
		2	3/4	75
Polyclonal	Vβ-Jβ-A	1	0/4	0
		2	0/4	0
	Vβ-Jβ-B	1	0/4	0
		2	0/4	0
	Dβ-Jβ	1	0/4	0
		2	0/4	0

Result (clonal *vs* polyclonal): Kappa= 0.76, SD=0.103 and CI 95% =0.55-0.96.

12.1.3 *Analytical specificity*

The analytical specificity was analyzed by testing the method with theoretical positive samples that imitated real clonal and polyclonal samples B and T. Genomic DNA extracted from fresh frozen tissue (tonsil) was used to construct the theoretical clonal positive samples, a control that presents a polyclonal pattern for all the fragments analyzed, mixed with 50% of DNA from cell lines.

DNAs from cell lines used in the study:

- RAMOS clonal cell line: cells derived from human Burkitt Lymphoma. Clonal positive control B.
- JURKAT clonal cell line: from T cells obtained from peripheral blood of the patient with acute lymphoblastic leukemia. Clonal positive control T.
- MOLT3 clonal cell line: cells derived from human acute lymphoblastic leukemia. Clonal positive control T.
- HUT 78 clonal cell line: cutaneous T cells from patient with Sézary syndrome. Clonal positive control T.

Genomic DNA extracted from fresh frozen tissue (tonsil) showing a polyclonal pattern for all fragments analyzed was used as polyclonal positive controls.
The samples used were prepared at a concentration such that a final amount of 0.2 µg of DNA per reaction could be obtained.
All results were analyzed by capillary electrophoresis with the Genemapper software. The interpretation of the results was carried out taking into account the indications of the latest updates of the EuroClonality/BIOMED-2 group guide for the interpretation of the results.

Specificity of TCR beta Rearrangements Molecular Analysis Kit		
	TCR rearrangement (TCRbeta)	**Rearrangement B (IgH and Ig K-L)**
Tonsil DNA+ 50% RAMOS cell line DNA	Polyclonal	Clonal
Tonsil DNA + 50% JURKAT cell line DNA	Clonal	Polyclonal
Tonsil DNA + 50% MOLT3 cell line DNA	Clonal	Polyclonal
Tonsil DNA + 50% HUT cell line DNA	Clonal	Polyclonal
Fresh tissue DNA (tonsil)	Polyclonal	Polyclonal

Table 10: Specificity test of TCR beta Rearrangements Molecular Analysis Kit

No cross-reactions between the different cell lines used as clonal positive controls B and T or polyclonal positive control were observed. In all cases, 100% specificity was obtained.

12.1.4 *Analytical sensitivity*

The three amplification mixes (VßJß-A, VßJß-B and DßJß) were tested with serial dilutions of DNA from the T lymphoma cell line with a fixed amount of DNA from peripheral blood lymphocytes to obtain a final amount of 0.2 µg DNA per reaction.

DNAs used in the study:

- Clonal cell line JURKAT1 (mix of amplification VßJß-A): Genomic DNA obtained from an immortalized cell line (JURKAT1) of T lymphocytes, obtained from ATCC which is molecularly characterized and reordered fragments analyzed in the kit.
- Clonal cell line MOLT3 (mix of amplification VßJß-B): Genomic DNA obtained from an immortalized cell line (MOLT3) of T lymphocytes, obtained from ATCC which is molecularly characterized and reordered fragments analyzed in the kit.
- Clonal cell line HUT78 (mix of amplification DßJß): Genomic DNA obtained from cutaneous lymphocytes T from the ATCC, which has been characterized at a molecular level and the DßJß fragment has been rearranged.
- Peripheral blood lymphocytes (PBL): Genomic DNA from control peripheral blood lymphocytes, which presents a polyclonal pattern for all the fragments analyzed.

% clonal DNA	**VßJß-A**		**VßJß-B**		**Dβ-Jβ**	
	Clonal Result	Sensitivity	Clonal Result	Sensitivity	Clonal Result	Sensitivity
100	10/10	100%	10/10	100%	10/10	100%
50	10/10	100%	10/10	100%	10/10	100%
20	10/10	100%	7/10	70%	10/10	100%
10	10/10	100%	5/10	50%	7/10	70%
5	8/10	80%	2/10	20%	3/10	30%
0	0/10	0%	0/10	0%	0/10	0%

Table 11: Results obtained with each of the amplification mixes for TCR beta according to the percentage of clonal DNA present in the sample.

Tests have shown that this method is capable of detecting 5 to 10 tumor cells in a population of 100 normal cells.

12.1.5 *Results of limit of detection:*

Mix	*Limit of detection*
VßJß-A	10 ng clonal DNA (5 % in 0.2 µg total)
VßJß-B	20 ng clonal DNA (10 % in 0.2 µg total)
Dβ-Jβ	20 ng clonal DNA (10 % in 0.2 µg total)

Table 12: Limit of detection for each of the amplification mixes included in the TCR beta Rearrangements Molecular Analysis Kit

12.1.6 *Diagnostic specificity and sensitivity*

The clinical performance of TCR beta Rearrangements Molecular Analysis Kit was validated from DNA extracted and purified with the extraction methods described in section 7 of this manual. The diagnostic capacity of the kit was evaluated by testing its diagnostic sensitivity and specificity. These two parameters are defined and calculated as follows:

- The **diagnostic specificity** is expressed as a percentage (numerical fraction multiplied by 100), calculated as 100 x the number of true negative values (TN) divided by the sum of true negative values (TN) plus the number of false positive (FP) values, or 100 × TN/ (TN + FP).

- The **diagnostic sensitivity** is expressed as a percentage (numerical fraction multiplied by 100), calculated as 100 × the number of true positive values (TP) divided by the sum of true positive values (TP) plus the number of false negative values (FN), or 100 × TP/ (TP + FN).

The DNA purified from 85 clinical samples was analyzed retrospectively. 21/85 clonal samples (28.2%) were detected with a percentage of global concordance between molecular result and diagnosis based on clinical-pathological and immunohistochemical data of 91% (Kappa=0.77).

T cell lymphoproliferative diseases	TN	FP	TP	FN	Diagnostic specificity	Diagnostic sensitivity
TCR beta gene rearrangements (MAD-003993TP-2)	56	3	21	5	94.9%	80.8%
TCR gamma gene rearrangements (MAD-003994TP-2)	55	4	24	2	93.2%	92.3%
TCR beta+ TCR gamma gene rearrangements (MAD-003993TP-2 and MAD-003994TP-2)	55	4	25	1	93.2%	96.2%

Table 13: Diagnostic sensitivity and specificity of TCR beta Rearrangements Molecular Analysis kit.

Conclusion: monoclonal amplification combining the three mixes of primers included in this kit (MAD-003993TP-2) is specific to the T lymphoid line and will allow detection of up to 80.8% of T-cell malignant lymphoproliferative processes.
The results also demonstrate the complementarity of the TCR beta and TCR gamma targets for the detection of T clonality. The combined analysis of rearrangements in the TCR beta and gamma TCR genes increases the number of clonal cases detected from 21 (TCR beta) to 25 (TCR beta+TCR gamma rearrangements) with a sensitivity of 96.2%.

13 LIMITATIONS

- A negative clonality result with this test does not exclude the diagnosis of lymphoma from other clinical, morphological, and immunophenotypic data.
- The detection of a clonal rearrangement pattern by this technique must be accompanied by an interpretation in conjunction with the rest of the clinical data.
- Tests have shown that this method is capable of detecting up to 5-10 tumor cells in a population of 100 normal cells.
- Blood samples: Do not use heparinized whole blood. It is recommended to use EDTA or Trisodium Citrate as anticoagulants
- Reagents that can inhibit PCR are used in bone marrow samples during the decalcification process.
- In samples from paraffin-embedded and fixed material it is important to control the type and time of fixation (buffered formalin) as this can affect the quality of the DNA and therefore the ability to amplify by PCR. DNA from these samples may also be affected if they come from biopsies archived for more than 2 years.

14 TROUBLESHOOTING

Problem	Causes	Solutions
No band/peaks in all the positive amplification controls.	Kit or any of its components expired or incorrectly stored.	Check the expiration date of all the reagents and the storage conditions. Repeat the test
	Problems in the amplification by PCR.	Check that the thermal cycler program is appropriate/ that both polymerase and positive control DNA have been added to the PCR mix/ that the PCR mix has not frozen after addition of the polymerase and repeat the assay.
	Problems in the use of the indicated clonal positive control	Check that the clonal positive control indicated for each PCR mix is used. DNA clonal positive control T JURKAT for the VßJß-A and DßJß mixes and clonal positive

		control T MOLT3 for the VßJß-B mix
No band/peaks in the internal amplification control of the test sample	Presence of PCR inhibitors. Not enough amount of human DNA in the clinical sample. Low quality/quantity of the DNA in the sample.	Verify the correct functioning of the extraction system of nucleic acids used/ repeat the PCR by diluting the starting sample and repeat the test. Repeat the DNA extraction starting from more sample/reduce the elution volume and repeat the test. Repeat the DNA extraction starting from a new sample and repeat the test.
Absence of band(s)/peak(s) for TCR beta gene amplification mixes in the presence of band/peak for the internal amplification control of the test sample.	Absence of test DNA/polymerase in the amplification mixes for TCR beta. Absence/low T-cellularity in the test sample	Repeat the test making sure that both polymerase and DNA from the test sample are added to each amplification mix for TCR beta and that the PCR mix is not frozen after the polymerase is added. Repeat the DNA extraction starting from more sample/ another sample/reducing the elution volume and repeat the test.
Signal too intense in capillary electrophoresis	Saturated/truncated peaks appear. The system is not able to measure the actual value of the signal.	Dilute sample prior to PCR/Load less PCR product/dilute PCR product/decrease sample injection time or voltage in electrophoresis

Low signal intensity in capillary electrophoresis	Little difference between specific signal and background noise	Increase sample amount/Concentrate sample/Increase sample injection time or voltage in electrophoresis.

Table 14. Possible incidents, causes and solutions to problems that may occur during the analysis.

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16 LABEL AND BOX SYMBOLS

IVD	In vitro diagnostic medical device		Expiration date
REF	Catalog number		Temperature limit
LOT	Lot code		Manufacturer
	Refer to the instructions for use		Sufficient content for <n> assays
	Safety data sheet		

17 GLOSSARY

DNA: Deoxyribonucleic acid.
ATCC: American Type Culture Collection
IC: Internal control.
DNase: Deoxyribonuclease.

dNTP: nucleoside triphosphate
EDTA: Ethylenediaminetetraacetic acid
EtBr: Ethidium bromide.
FN: False negative.
FP: False positive False positive.
g: relative centrifugal force or g force (RCF).
h: hour(s).
CI: confidence interval
Ig: Immunoglobulin.
IgH: Immunoglobulin heavy chain
IgK: Immunoglobulin kappa light chain
IgL: Immunoglobulin lambda light chain
Min: minutes.
MW: molecular weight
pb: Base pairs.
PBL: Peripheral blood lymphocytes. Peripheral blood lymphocytes.
PBS: Phosphate buffered saline. Phosphate buffered saline.
PCR: Polymerase Chain Reaction.
RFU: Relative fluorescence units.
RNase: Ribonuclease.
RPM: Revolution per minute.
SD: Standard deviation. Standard deviation.
Sec: Seconds.
TBE: Tris/borate/EDTA buffer.
TCR: T cell receptor. T-cell receptor.
TE: Tris-EDTA buffer.
TN: True negative. True negative results.
TP: True positive. True positive results.
TN: True negative.
TP: true positive.
6-FAM: 6-carboxyfluorescein.

18 CHANGELOG

Date	Description
2020-06-23	• Update of the interpretation of results including representative images.
2024-01-29	• New structure of the IFU format • Addition of section "Contents" • Addition of section “Intended use” • Addition of section “Principle of the method” • Update of section “Warnings and precautions” • Update of section “Storage and stability conditions” • Update of section “Analysis of analyzed products” • Addition of section “Quality control procedure”

	• Update of section “Interpretation of results” • Addition of section “Performance characteristics” • Update of section “Limitations” • Addition of section “Troubleshooting” • Addition of section “Label and box symbols” • Addition of section "Glossary" • Addition of section “Changelog”