IdentiClone®

Instructions for Use

# IdentiClone® *TCRB* Gene Clonality Assay

CE UK CA IVD

For identification of clonal T cell receptor beta chain gene rearrangements.

IVD For *In Vitro* Diagnostic Use

invivoscribe

Storage Conditions: **-85ºC to -65ºC**
(DNA controls may be separated from assay kits and stored at 2°C to 8°C)

| Catalog # | Product | Quantity |
|---|---|---|
| REF 92050011 | IdentiClone *TCRB* Gene Clonality Assay – ABI Fluorescence Detection | 33 Reactions |
| REF 92050021 | IdentiClone *TCRB* Gene Clonality Assay MegaKit – ABI Fluorescence Detection | 330 Reactions |

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# 1. Intended Use

The IdentiClone TCRB Gene Clonality Assay is an in vitro diagnostic product intended for PCR-based detection of clonal T-cell receptor beta chain gene rearrangements in patients with suspect lymphoproliferations. Specifically, the *TCRB* Gene Clonality Assay can be used to:

- Identify clonality in suspect lymphoproliferations
- Support a differential diagnosis between reactive lesions and T-cell and some immature B-cell malignancies
- Determine lineage involvement in mature lymphoproliferative disorders
- Monitor and evaluate disease recurrence

# 2. Summary and Explanation of the Test

## 2.1. Background

Rearrangements of the antigen receptor genes occur during ontogeny in B and T lymphocytes. These gene rearrangements generate products that are unique in length and sequence for each cell. Therefore, polymerase chain reaction (PCR) assays can be used to identify lymphocyte populations derived from a single cell by detecting the unique V-J gene rearrangements present within these antigen receptor loci.1 This IdentiClone PCR assay employs multiple consensus DNA primers that target conserved genetic regions within the T cell receptor beta chain gene. This test is used to detect the vast majority of clonal T-cell malignancies from DNA. Test products can be analyzed using a variety of detection formats, including gel and capillary electrophoresis.

Gene rearrangement analysis can also be performed by Southern Blot (SB)-based techniques. Although SB analysis is very reliable, it is increasingly replaced by PCR techniques because of the greater efficiency and sensitivity of PCR. Moreover, PCR is relatively easy, less labor intensive and requires much lower quantities of high molecular weight DNA than SB tests. In addition, PCR can often be performed on DNA isolated from paraffin-embedded tissue, whereas SB cannot be performed because the DNA is often degraded. Therefore, there is a strong need to replace SB analysis with reliable PCR techniques.

## 2.2. Summary

Invivoscribe's IdentiClone assays represent a new approach to PCR-based clonality testing. These standardized assays were carefully optimized testing positive and negative control samples using multiplex master mixes. Assay development was followed by extensive validation including the testing of more than 400 clinical samples using Revised European/American Lymphoma (REAL) Classification. Testing was done at more than thirty prominent independent testing centers throughout Europe in a collaborative study known as the BIOMED-2 Concerted Action. Results from this BIOMED-2 study appear in Leukemia, a leading peer-reviewed journal.[2]

The ABI detection-based assays cannot reliably detect clonal populations comprising less than 1% of the total lymphocyte cell population. Always interpret the results of molecular clonality tests in the context of clinical, histological and immunophenotypic data.

This test kit includes 4 master mixes. *TCRB* Tubes A and B target framework regions within the variable region and the joining region of the *TCR* beta chain locus. *TCRB* Tube C targets the diversity and joining regions. Lastly, the Specimen Control Size Ladder master mix, targets multiple genes and generates a series of amplicons of 96, 197, 297, 397 and 602 base pairs to ensure that the quality and quantity of input DNA is adequate to yield a valid result. A single thermal cycler program and similar detection methodologies are used with all of our Gene Clonality Assays. This improves consistency and facilitates cross training on a broad range of different assays.

This assay is based on the Euroclonality/BIOMED-2 Concerted Action BMH4-CT98-3936.

# 3. Principles of the Procedure

## 3.1. Polymerase Chain Reaction (PCR)

PCR assays are routinely used for the identification of clonal T-cell populations. These tests amplify the DNA between primers that target the conserved variable (V) regions and the conserved joining (J) regions (*TCRB* Tubes A and B), as well as the diversity (D) and joining regions (*TCRB* Tube C). These conserved regions lie on either side of an area within the V-J region where programmed genetic rearrangements occur during maturation of all B and T lymphocytes. The antigen receptor genes that undergo rearrangement are the immunoglobulin heavy chain and light chains in B-cells and the T cell receptor genes in T-cells. Each B- and T-cell has a single productive V-J rearrangement that is unique in both length and sequence. Therefore, when DNA from a normal or polyclonal population is amplified using DNA primers that flank the V-J region, a bell-shaped curve (Gaussian distribution) of amplicon products within an expected size range is produced. On a gel, this distribution of products is seen as a smear. This Gaussian distribution reflects the heterogeneous population of V-J rearrangements. (In certain cases, where lymphocyte DNA is not present, no product is seen.) For DNA from samples containing a clonal population, the yield is one or two prominent amplified products (amplicons) within a diminished polyclonal background.

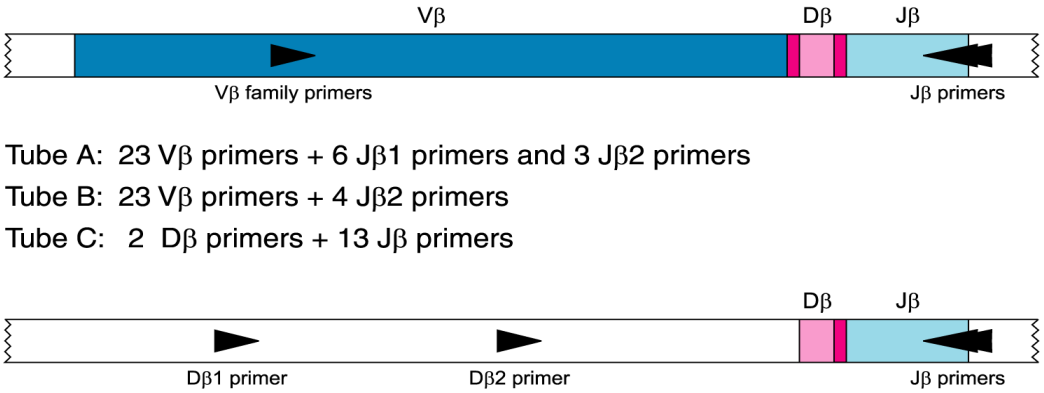


**Figure 1**. This is a simplified diagram of a representative rearranged T cell receptor beta gene on chromosome 7 (7q35) showing the approximate placement of the upstream and downstream DNA primers. The numbers of primers and their specificity are listed for master mix Tubes A, B and C.

Since the antigen receptor genes are polymorphic (consisting of a heterogeneous population of related DNA sequences), it is difficult to employ a single set of DNA primer sequences to target all of the conserved flanking regions around the V-J rearrangement. N-region diversity and somatic mutation further scramble the DNA sequences in these regions. Therefore, multiplex master mixes, which target several FR regions, are required to identify the majority of clonal rearrangements. As indicated, clonal rearrangements are identified as prominent, single-sized products within the background of different-sized amplicon products that form a Gaussian distribution around a statistically favored, average-sized rearrangement.

## 3.2. Differential Fluorescence Detection

Differential fluorescence detection is commonly used to resolve the different-sized amplicon products using a capillary electrophoresis instrument. Primers can be conjugated with several different fluorescent dyes (fluorophores) so that they can produce different emission spectra upon excitation by a laser in the capillary electrophoresis instrument. In this manner, different fluorescent dyes can correspond to different targeted regions. This detection system results in unsurpassed sensitivity, single nucleotide resolution, differential product detection and relative quantification. In addition, the use of agarose and polyacrylamide gels, as well as the use of carcinogens such as ethidium bromide, can virtually be eliminated. Further, differential detection allows accurate, reproducible and objective interpretation of primer-specific products and automatic archiving of data. Inter-assay and intra-assay reproducibility in size determination using capillary electrophoresis is approximately 1 to 2 base pair. This reproducibility and sensitivity coupled with the automatic archiving of specimen data allows for the monitoring, tracking and comparison of data from individual patients over time.

# 4. Reagents

## 4.1. Reagent Components

Table 1. Available Kits

| Catalog # | Product Name | Quantity |
|---|---|---|
| REF 92050011 | IdentiClone *TCRB* Gene Clonality Assay – ABI Fluorescence Detection | 33 Reactions |
| REF 92050021 | IdentiClone *TCRB* Gene Clonality Assay MegaKit – ABI Fluorescence Detection | 330 Reactions |

Table 2. Reagent Components

| Reagent | Catalog # | Reagent Components (active ingredients) | Unit Quantity | 92050011 # of Units | 92050021 # of Units | Storage Temp. |
|---|---|---|---|---|---|---|
| Master Mixes | 22050011CE | ***TCRB* Tube A – 6FAM & HEX**<br>Multiple oligonucleotides targeting the Vβ + Jβ1 + Jβ2 regions of the T cell receptor beta gene in a buffered salt solution. | 1500 µL | 1 | 10 | -85°C to -65°C |
| | 22050021CE | ***TCRB* Tube B – 6FAM**<br>Multiple oligonucleotides targeting the Vβ + Jβ2 regions of the T cell receptor beta gene in a buffered salt solution. | 1500 µL | 1 | 10 | |
| | 22050031CE | ***TCRB* Tube C – 6FAM & HEX**<br>Multiple oligonucleotides targeting the Dβ + Jβ1 + Jβ2 regions of the T cell receptor beta gene in a buffered salt solution. | 1500 µL | 1 | 10 | |
| Template Amplification Control Master Mix | 20960021 | **Specimen Control Size Ladder – 6FAM**<br>Multiple oligonucleotides targeting housekeeping genes. | 1500 µL | 1 | 10 | |
| Positive Control DNAs | 40880490 | **IVS-0009 Clonal Control DNA**<br>200 µg/mL of DNA in 1/10$^{th}$ TE solution | 100 µL | 1 | 5 | 2°C to 8°C or -85°C to -65°C |
| | 40880190 | **IVS-0004 Clonal Control DNA**<br>200 µg/mL of DNA in 1/10$^{th}$ TE solution | 100 µL | 1 | 5 | |
| | 40881210 | **IVS-0021 Clonal Control DNA**<br>200 µg/mL of DNA in 1/10$^{th}$ TE solution | 100 µL | 1 | 5 | |
| Negative (Normal) Control DNA | 40920010 | **IVS-0000 Polyclonal Control DNA**<br>200 µg/mL of DNA in 1/10$^{th}$ TE solution | 100 µL | 1 | 5 | |

Note: There are no preservatives used in the manufacture of this kit.

## 4.2. Warnings and Precautions

- IVD This product is for in vitro diagnostic use
- Use the assay kit as a system; do not substitute other manufacturer's reagents. Dilution, reducing amplification reaction volumes or other deviation in this protocol may affect the performance of this test and/or nullify any limited sublicense that comes with the purchase of this testing kit.
- Materials are stable until the labeled expiration date when stored and handled as directed. Do not use kits beyond their expiration date.
- Close adherence to the protocol will assure optimal performance and reproducibility. Ensure correct thermal cycler program is used, as other programs may provide inaccurate/faulty data, such as false positive and false negative results.
- Do not mix or combine reagents from kits with different lot numbers.
- Laboratory personnel are reminded to wear appropriate personal protective equipment and follow good laboratory practices and universal precautions when working with specimens. Handle specimens in approved biological safety containment facilities and open only in certified biological safety cabinets. Use molecular biology grade water for the preparation of specimen DNA.
- Due to the analytical sensitivity of this test, use extreme care to avoid the contamination of reagents or amplification mixtures with samples, controls or amplified materials. Closely monitor all reagents for signs of contamination (e.g., negative controls giving positive signals). Discard reagents suspected of contamination.
- To minimize contamination, wear clean gloves when handling samples and reagents and routinely clean work areas and pipettes prior to doing PCR.
- Autoclaving does not eliminate DNA contamination. Follow uni-directional work flow separate work areas in the PCR laboratory: begin with Master Mix Preparation, move to the Specimen Preparation, then to the Amplification and finally to Detection. Do not bring amplified DNA into the areas designated for master mix or specimen preparation.
- Use all pipettes, pipette tips and any equipment used in a particular area to that area of the laboratory.
- Use sterile, disposable plastic ware whenever possible to avoid RNase, DNase or cross-contamination.

## 4.3. Storage and Handling

- For any duration other than immediate use, store assay kits at -85°C to -65°C.
- The optimum storage temperature for DNA controls is 2°C to 8°C, but DNA controls can be stored at -85°C to -65°C.
- All reagents and controls must be thawed and vortexed or mixed thoroughly prior to use to ensure that they are resuspended completely. Excessive vortexing may shear DNA and cause labeled primers to lose their fluorophors.
- Materials are stable until the labeled expiration date when stored and handled as directed. Do not use kits beyond their expiration date.
- Due to high salt concentrations, PCR master mixes are sensitive to freeze/thaw cycles. Aliquot master mixes into sterile o-ring screw-cap tubes if necessary.

# 5. Instruments

## 5.1. Thermal cycler

- Use or Function: Amplification of DNA samples
- Suggested Instrument: Veriti™ thermal cycler or equivalent
- Performance characteristics and specification:
    - Minimum Thermal Range: 15°C to 96°C
    - Minimum Ramping Speed: 0.8°C/sec
- Follow manufacturer's installation, operation, calibration and maintenance procedures.
- See section 7.4 *Amplification* for thermal cycler program.

## 5.2. ABI Capillary Electrophoresis Instruments

- Use or Function: Fragment detection and analysis
- The following capillary electrophoresis instruments will meet the performance needs for this assay:
    - ABI 310 Genetic Analyzer (1-capillary)
    - ABI 3100 Avant Genetic Analyzer (4-capillaries)
    - ABI 3100 Genetic Analyzer (16-capillaries)
    - ABI 3130 Genetic Analyzer (4-capillaries)
    - ABI 3130xL Genetic Analyzer (16-capillaries)
    - ABI 3500 Genetic Analyzer (8-capillaries)
    - ABI 3500xL Genetic Analyzer (24-capillaries)
- Follow manufacturer's installation, operation, calibration and maintenance procedures.
- The ABI instrument used must be calibrated with appropriate Matrix Standards as outlined in section 7.2 *Materials Required (not provided)*.
- Use the default settings for your polymer and capillary type.
- See section 7.5 *ABI Fluorescence Detection* for sample preparation.

# 6. Specimen Collection and Preparation

## 6.1. Precautions

Biological specimens from humans may contain potentially infectious materials. Handle all specimens in accordance with the OSHA Standard on Bloodborne Pathogens or Biosafety Level 2.

## 6.2. Interfering Substances

The following substances are known to interfere with PCR:

- Divalent cation chelators
- Low retention pipette tips
- EDTA (not significant at low concentrations)
- Heparin

## 6.3. Specimen Requirements and Handling

This assay tests **genomic DNA** from the following sources:

- 5 cc of peripheral blood, bone marrow biopsy or bone marrow aspirate anti-coagulated with heparin or EDTA (stored at 2°C to 8°C and shipped at ambient temperature)
- Minimum 5 mm cube of tissue (stored and shipped frozen; or stored and shipped in RPMI 1640 at ambient temperature or on ice)
- 2 µg of genomic DNA (stored at 2°C to 8°C and shipped at ambient temperature)
- Formalin-fixed paraffin embedded tissue or slides (stored and shipped at ambient temperature)

## 6.4. Sample Preparation

Extract the genomic DNA from patient specimens as soon as possible. Resuspend DNA to a final concentration of 100 µg to 400 µg per mL in 1/10th TE (1 mM Tris-HCl, pH 8.0; 0.1 mM EDTA) or in molecular biology grade or USP water. This is a robust assay system. A wide range of DNA concentrations will generate a valid result. Therefore, quantifying and adjusting DNA concentrations is generally not necessary. Testing sample DNA with the Specimen Control Size Ladder master mix will ensure that DNA of sufficient quality and quantity was present to yield a valid result.

## 6.5. Sample Storage

Store genomic DNA at 2°C to 8°C or at -85°C to -65°C until use.

# 7. Assay Procedure

## 7.1. Reagent Components

Table 3. Materials Provided

| Catalog # | Description |
|---|---|
| 22050011CE | *TCRB* Tube A – 6FAM |
| 22050021CE | *TCRB* Tube B – 6FAM |
| 22050031CE | *TCRB* Tube C – 6FAM & HEX |
| 20960021 | Specimen Control Size Ladder – 6FAM |
| 40880490 | IVS-0009 Clonal Control DNA |
| 40880190 | IVS-0004 Clonal Control DNA |
| 40881210 | IVS-0021 Clonal Control DNA |
| 40920010 | IVS-0000 Polyclonal Control DNA |

## 7.2. Materials Required (not provided)

Table 4. Materials Required (not provided)

| Reagent/Material | Recommended Reagents/Materials and Suppliers | Catalog # | Notes |
|---|---|---|---|
| **DNA Polymerase** | Roche:<br>• EagleTaq DNA Polymerase<br>Invivoscribe:<br>• FalconTaq DNA Polymerase<br>or equivalent | 05206944190<br><br>60970130 | N/A |
| **Glass Distilled De-ionized Molecular Biology Grade or USP Water** | N/A | N/A | DNase / RNase free |
| **Calibrated Pipettes** | Rainin:<br>• P-2, P-20, P-200 and P-1000 pipettes<br>• Or SL-2, SL-20, SL-200 and SL-1000 pipettes | N/A | Must be able to accurately measure volumes between 1 µL and 1000 µL. |
| **Thermal cycler** | Thermo Fisher Scientific:<br>• Veriti Dx Thermal Cycler<br>Bio-Rad:<br>• MJ Research PTC-100 or PTC-200, PTC-220, PTC-240<br>Perkin-Elmer<br>• PE 9600 or PE 9700 | N/A | N/A |
| **Vortex Mixer** | N/A | N/A | N/A |
| **PCR plates or tubes** | N/A | N/A | Sterile |
| **Filter barrier pipette tips** | N/A | N/A | Sterile, RNase/DNase/ Pyrogen-free |
| **Microcentrifuge tubes** | N/A | N/A | Sterile |
| **ABI Capillary Electrophoresis Instrument** | Thermo Fisher Scientific:<br>• ABI 310, 3100 or 3500 series | N/A | N/A |
| **Hi-Di Formamide** | Thermo Fisher Scientific:<br>• Hi-Di™ Formamide | 4311320 | N/A |
| **Size Standards** | Invivoscribe:<br>• Hi-Di Formamide w/ROX size standards for ABI 3100<br>Thermo Fisher Scientific:<br>• For ABI 3100 or 3130 instruments:<br>o GeneScan™ - 400HD [ROX]™<br>• For ABI 3500 instruments:<br>o GeneScan™ - 600 [LIZ]™ v2.0 | 60980061<br><br>402985<br><br>4408399 | N/A |

Table 4. Materials Required (not provided)

| Reagent/Material | Recommended Reagents/Materials and Suppliers | Catalog # | Notes |
|---|---|---|---|
| **Spectral Calibration Dye Sets** | Thermo Fisher Scientific:<br>• For ABI 3100 and 3130 instruments:<br>o DS-30 Matrix Standard Kit (Dye Set D)<br>• For ABI 310 instruments:<br>o NED Matrix Standard<br>o And Fluorescent Amidite Matrix Standards [6FAM, TET, HEX, TAMRA, ROX]<br>• For ABI 3500 instruments:<br>o DS-33 Matrix Standard Kit (Dye Set G5) | <br><br>4345827<br><br>402996<br>401546<br><br>4345833 | N/A |
| **Polymer** | Thermo Fisher Scientific:<br>• POP-4™ Polymer:<br>o POP-4 for 310 Genetic Analyzers<br>o POP-4 for 3100/3100-Avant Genetic Analyzers<br>o POP-4 for 3130/3130xL Genetic Analyzers<br>• POP-7™ Polymer:<br>o POP-7 for 3130/3130xL Genetic Analyzers<br>o POP-7 for 3500/3500xL Genetic Analyzers | <br><br>402838<br>4316355<br>4352755<br><br>4352759<br>4393714 | N/A |
| **Buffer** | Thermo Fisher Scientific:<br>• 10X Genetic Analyzer Buffer with EDTA | <br>402824 | Dilute 1:10 in sterile water prior to use. |

## 7.3. Reagent Preparation

- Testing unknown samples using the Specimen Control Size Ladder master mix can ensure that no inhibitors of amplification are present and there is DNA of sufficient quality and quantity to generate a valid result.
- Singlicate test results are valid; however, we recommend duplicate testing when possible. If duplicate testing provides inconsistent results, re-testing or re-evaluation of the sample is necessary.
- Test positive, negative and no template controls for each master mix.
- Batch multiple samples in a run to avoid running out of the negative control (IVS-0000 Polyclonal Control DNA). If batching samples is not practical in your laboratory setting, IVS-0000 Polyclonal Control DNA is also available for purchase separately.

7.3.1. Using gloved hands, remove the master mixes from the freezer. Allow the tubes to thaw completely; then gently vortex to mix.

7.3.2. In containment hood or dead air box remove an appropriate aliquot from each master mix to individual clean, sterile microcentrifuge tubes.

- Aliquot volumes are 45 µL for each reaction.
- Add an additional reaction for every 15 reactions to correct for pipetting errors. Thus, for each master mix (except for the Specimen Control Size Ladder), the number of reactions (n) is:

| | | |
|---|---|---|
| n = | 2 × # of samples | (run each sample in duplicate) |
| | + 1 | positive control DNA (See Table 6) |
| | + 1 | negative control DNA (IVS-0000 Polyclonal Control DNA) |
| | + 1 | no template control (water) |
| | + 1 | to correct for pipetting errors |
| n = | 2 × # of samples + 4 | Total |

- Therefore the total aliquot volume for each master mix is **n × 45 µL**.
- For the Specimen Control Size Ladder master mix, the number of reactions (**m**) is:

| | | |
|---|---|---|
| m = | # of samples | (run each sample in singlicate) |
| | + 1 | positive control DNA (IVS-0000 Polyclonal Control DNA) |
| | + 1 | no template control (water) |
| | + 1 | to correct for pipetting errors |
| m = | # of samples + 3 | Total |

- Therefore the total aliquot volume for the Specimen Control Size Ladder master mix is **m × 45 µL**.

7.3.3. **For *TCRB* Tubes A and B:** Add 2.25 U (or 0.45 µL @5 U/µL) of Taq DNA polymerase per reaction to each master mix.

- The total Taq DNA polymerase added to each master mix is **n × 0.45 µL**.
- Gently vortex to mix.

7.3.4. **For *TCRB* Tube C and Specimen Control Size Ladder:** Add 1.25 U (or 0.25 µL @5 U/µL) of Taq DNA polymerase per reaction.

- The total Taq DNA polymerase added to each master mix is **n × 0.25 µL** for the *TCRB* Tube C master mix and **m × 0.25 µL** for the Specimen Control Size Ladder master mix.
- Gently vortex to mix.

7.3.5. For each reaction, aliquot 45 µL of the appropriate master mix + DNA polymerase solution into individual wells in a PCR plate or tube.

7.3.6. Add 5 µL of appropriate template (sample DNA, positive control DNA, negative control DNA or water) to the individual wells containing the respective master mix solutions. Pipette up and down several times to mix.

7.3.7. Cap or cover the PCR plate.

- Samples are now ready to be amplified on a thermal cycler.

7.3.8. If amplification cannot be performed immediately following reagent preparation, the PCR plate or tubes can be stored at 2°C to 8°C for up to 24 hours.

**Quick Guide**

For each master mix and n reactions, mix:

| | |
|---|---|
| **n × 45 µL** | Master Mix |
| **n × 0.25 µL or 0.45 µL*** | Taq DNA polymerase |

Vortex gently to mix.

Aliquot 45 µL of master mix + DNA polymerase solution into each reaction well.

Add 5 µL of appropriate Template to each well.

| | |
|---|---|
| **Total reaction volume =** | **50 µL** |

*Note: Use 0.45 µL of Taq DNA polymerase for *TCRB* Tubes A and B and 0.25 µL of Taq DNA polymerase for *TCRB* Tube C and Specimen Control Size Ladder

## 7.4. Amplification

7.4.1. Amplify the samples using the following PCR program:

- Use the **calculated** option for temperature measurement with the BioRad MJ Research PTC thermal cyclers.

Table 5. Thermal cycling conditions

| Step | Temperature | Duration | Cycles |
|---|---|---|---|
| 1 | 95°C | 7 minutes | 1 |
| 2 | 95°C | 45 seconds | 35 |
| 3 | 60°C | 45 seconds | |
| 4 | 72°C | 90 seconds | |
| 5 | 72°C | 10 minutes | 1 |
| 6 | 15°C | ∞ | 1 |

7.4.2. Remove the amplification plate or tubes from the thermal cycler.

7.4.3. Although amplified DNA is stable at room temperature for extended periods of time, store PCR products at 2°C to 8°C until detection. Detection must be within 30 days of amplification.

## 7.5. ABI Fluorescence Detection

Do not multiplex PCR products from different master mixes, as this will result in overall reduced sensitivity of the assay.

### ABI 310, 3100, or 3130 Platforms.

7.5.1. In a new microcentrifuge tube, mix an appropriate amount (for a total of 10 µL per reaction) of Hi-Di Formamide with ROX Size Standards.[a] Vortex well.
7.5.2. In a new 96-well PCR plate, add 10 µL of Hi-Di Formamide with ROX size standards to individual wells for each reaction.
7.5.3. Transfer 1 µL of each reaction to the wells containing Hi-Di Formamide with ROX size standards. Add only one sample per well. Pipette up and down to mix.
7.5.4. Cap or cover the PCR plate or tubes.
7.5.5. Heat denature the samples at 95ºC for 2 minutes then snap chill on ice for 5 minutes.
7.5.6. Prepare a **sample sheet** and **injection list** for the samples.
7.5.7. Run the samples on an ABI capillary electrophoresis instrument according to the user manual.
7.5.8. Review profile and controls, report results. (see sections 8 *Interpretation of Results* and 10 *Expected Values*).

- Data are automatically displayed as size and color specific peaks.

### ABI 3500 Platforms:

Note: Due to instrument to instrument variation in the performance of the ABI 3500 platform, the amount of formamide, sample and size standard listed in the protocol is intended to be a starting point. The protocol may need to be optimized for specific ABI 3500 Platforms.

7.5.9. In a new microcentrifuge tube, mix an appropriate amount (9.5 µL per reaction) of Hi-Di Formamide with LIZ Size Standards.[a] Vortex well.
7.5.10. In a new 96-well PCR plate, add 9.5 µL of Hi-Di Formamide with LIZ size standards to individual wells for each reaction.
7.5.11. Transfer 0.5 µL of each reaction to the wells containing Hi-Di Formamide with LIZ size standards. Add only one sample per well. Pipette up and down to mix.
7.5.12. Cap or cover the PCR plate.
7.5.13. Heat denature the samples at 95ºC for 3 minutes, then snap chill on ice for 5 minutes.
7.5.14. Prepare a **sample sheet** and **injection list** for the samples.
7.5.15. Run the samples on an ABI 3500 capillary electrophoresis instrument according to its user manual.
7.5.16. Data are automatically displayed as size and color specific peaks. Review profile and controls, report results. (See sections 8 *Interpretation of Results* and 10 *Expected Values*)

[a]Note: Please see the Applied Biosystems product insert for mixing Hi-Di Formamide with size standards for different ABI instruments.

## 7.6. Quality Control

Positive and negative (or normal) controls are furnished with the kit and are run in singlicate each time the assay is performed to ensure proper performance of the assay. In addition, include a no template control (*e.g.* water) to test for contamination of the master mix or cross-contamination of reactions due to improper sterile technique. A buffer control may also be added to ensure that no contamination of the buffer used to resuspend the samples has occurred. The values for the positive controls are provided in Table 6. Additional controls and sensitivity controls (dilutions of positive controls into our negative control) are available from Invivoscribe.

### 7.7. Recommended Positive Controls

The amplicon sizes listed were determined using an ABI platform. Amplicon sizes seen on each capillary electrophoresis instrument may differ 1 to 4 bp from those listed depending on the platform of detection and the version of the analysis software used. Once identified, the amplicon size as determined on your specific platform will be consistent from run to run. This reproducibility is extremely useful when monitoring disease recurrence.

Note: "Color" indicates the color of products generated with the master mix when using the default color assignment on ABI fluorescence detection systems.

Table 6. Recommended Positive Controls

| Master Mix | Target | Color | Control DNA | Catalog # | Product Size (bp) |
|---|---|---|---|---|---|
| *TCRB* Tube A | Vβ + Jβ1/2 | Blue (Jβ2.X) + Green (Jβ1.X) | **Valid Size Range**<br>IVS-0009 Clonal Control DNA | ---<br>40880490 | **240 - 285**<br>264 |
| *TCRB* Tube B | Vβ + Jβ2 | Blue (Jβ2.X) | **Valid Size Range**<br>IVS-0004 Clonal Control DNA<br>IVS-0021 Clonal Control DNA | ---<br>40880190<br>40881210 | **240 - 285**<br>253<br>267 |
| *TCRB* Tube C | Dβ + Jβ1/2 | Blue (Jβ2.X) + Green (Jβ1.X) | **Valid Size Range**<br>IVS-0009 Clonal Control DNA | ---<br>40880490 | **170 - 210 (Dβ2), 285 - 325 (Dβ1)**<br>309 |
| Specimen Control Size Ladder | Multiple Genes | Blue | **Valid Size Range**<br>IVS-0000 Polyclonal Control DNA | ---<br>40920010 | **96, 197, 297, 397. 602**[a]<br>96, 197, 297, 397, 602[a] |

[a]Note: Because smaller PCR fragments are preferentially amplified, it is not unusual for the 602 bp fragment to have a diminished signal or to be missing entirely. For ABI fluorescence detection the 602 bp peak may not appear during normal run times. In addition, the size of this peak may differ by over 30 bp when fragment size is extrapolated using the GeneScan - 400HD [ROX] size standards.

# 8. Interpretation of Results

Although positive results are highly suggestive of malignancy, interpret both positive and negative results in the context of all clinical information and laboratory test results. The size range for each master mix has been determined by testing positive and negative control samples. For accurate and meaningful interpretation it is important to ignore peaks that occur outside of the valid size range for each master mix.

### 8.1. Analysis

8.1.1. Report samples that fail to amplify following repeat testing as "A result cannot be reported on this specimen because there was DNA of insufficient quantity or quality for analysis".

8.1.2. Repeat samples that test negative if the positive control reaction failed.

8.1.3. If samples run in duplicate yield differing results, re-test and/or re-evaluate the samples for sample switching.

8.1.4. All assay controls must be examined prior to interpretation of sample results.

Table 7. The following describes the analysis of each of the controls and the decisions necessary based upon the results.

| Type of Control | Expected Result | Aberrant Result |
|---|---|---|
| **No Template Control** | No amplification present, continue with analysis | Amplification present, Repeat the assay. |
| **Polyclonal Control** | Product size is consistent with expected size listed in section 10.1: *Expected Size of Amplified Products*. No clonal rearrangements are present. Continue with analysis. | Clonal rearrangements are present. Repeat the assay |
| **Positive Control**<br>(This can also be an extraction control if positive control material is taken through extraction processes) | Product size is consistent with expected size listed in section 10.1: *Expected Size of Amplified Products*. Continue with analysis. | Product not within expected size range. Repeat the assay. |
| **Specimen Control Size Ladder**<br>(This amplification control is essential for samples of unknown quantity and quality.) | If all of the 96, 197, 297, 397 and 602 bp peaks are seen, continue with analysis. Because smaller PCR fragments are preferentially amplified, it is not unusual for the 602 bp fragment to have a diminished signal or to be missing entirely. Continue with analysis. | If no bands are seen, repeat the assay unless specimen tests positive. If only 1, 2, or 3 bands are seen, re-evaluate sample for DNA degradation unless specimen tests positive. |

### 8.2. Sample Interpretation

Given that the controls produce expected results, interpret the clinical samples as follows:

- One or two prominent positive peaks[a] within the valid size range are reported as:

  "**Positive for the detection of clonal T cell receptor beta gene rearrangement(s) is consistent with the presence of a clonal cell population. In the context of overall diagnostic criteria, clonal cell populations can indicate the presence of hematologic malignancy.**"

- An absence of positive peaks[a] within the valid size range is reported as:

  "**Negative for the detection of clonal T cell receptor beta gene rearrangement(s)**."

[a]Note: Criteria for defining a positive peak are as follows:

- Products generated from **diagnostic samples** that fall within the valid size range and are at least three times the amplitude of the third largest peak in the polyclonal background are consistent with a positive peak.
- Products generated from **samples collected after initial diagnosis** that fall within the valid size range and are either; 1) at least three times the amplitude of the third largest peak; or, 2) exceed the amplitude of adjacent neighboring peaks and are identical in size to clonal amplicon products previously generated from the same patient using the same master mix, are consistent with a positive peak.

## 9. Limitations of Procedure

- This assay does not identify 100% of clonal cell populations.
- This assay cannot reliably detect less than one positive cell per 100 normal cells.
- Always interpret the results of molecular clonality tests in the context of clinical, histological and immunophenotypic data.
- PCR-based assays are subject to interference by degradation of DNA or to inhibition of PCR due to EDTA, heparin and other agents.

## 10. Expected Values

### 10.1. Expected Size of Amplified Products

The amplicon sizes listed were determined using an ABI platform. Amplicon sizes seen on your specific capillary electrophoresis instrument may differ 1 to 4 bp from those listed depending on the platform of detection and the version of the analysis software used. Once identified, the amplicon size as determined on your specific platform will be consistent from run to run. This reproducibility is extremely useful when monitoring disease recurrence.

Note: "Color" indicates the color of products generated with the master mix when using the default color assignment on ABI fluorescence detection systems.

Table 8. Expected Size of Amplified Products

| Master Mix | Target | Color | Control DNA | Catalog # | Product Size (bp) |
|---|---|---|---|---|---|
| ***TCRB* Tube A** | Vβ + Jβ1/2 | Blue (Jβ2.X) + Green (Jβ1.X) | **Valid Size Range**<br>IVS-0000 Polyclonal Control DNA<br>IVS-0009 Clonal Control DNA<br>IVS-0004 Clonal Control DNA | ---<br>40920010<br>40880490<br>40880190 | **240 - 285**<br>240 - 285, 271[a]<br>264<br>295 |
| ***TCRB* Tube B** | Vβ + Jβ2 | Blue (Jβ2.X) | **Valid Size Range**<br>IVS-0000 Polyclonal Control DNA<br>IVS-0009 Clonal Control DNA<br>IVS-0004 Clonal Control DNA<br>IVS-0021 Clonal Control DNA | ---<br>40920010<br>40880490<br>40880190<br>40881210 | **240 - 285**<br>240 - 285, 221[b]<br>---<br>253<br>267 |
| ***TCRB* Tube C** | Dβ + Jβ1/2 | Blue (Jβ2.X) + Green (Jβ1.X) | **Valid Size Range**<br>IVS-0000 Polyclonal Control DNA<br>IVS-0009 Clonal Control DNA<br>IVS-0004 Clonal Control DNA | ---<br>40920010<br>40880490<br>40880190 | **170 - 210 (Dβ2), 285 - 325 (Dβ1)**<br>128[b], 170-210, 285-325, 337[b]<br>309<br>295 |

Table 8. Expected Size of Amplified Products

| Master Mix | Target | Color | Control DNA | Catalog # | Product Size (bp) |
|---|---|---|---|---|---|
| **Specimen Control Size Ladder** | Multiple Genes | **Blue** | **Valid Size Range**<br>IVS-0000 Polyclonal Control DNA | ---<br>40920010 | **96, 197, 297, 397, 602**[c]<br>96, 197, 297, 397, 602[c] |

[a]Note: The 271 bp band is particularly seen in samples with low numbers of contaminating lymphoid cells.
[b]Note: Under sub-optimal conditions specific products of 221bp (in Tube B) and 128 and 337 bp (in Tube C) can be detected. If present, these bands will normally be faint.
[c]Note: Because smaller PCR fragments are preferentially amplified, it is not unusual for the 602 bp fragment to have a diminished signal or to be missing entirely. For ABI fluorescence detection the 602 bp peak may not appear during normal run times. In addition, the size of this peak may differ by over 30 bp when fragment size is extrapolated using the GeneScan - 400HD [ROX] size standards

## 10.2. Sample Data

The data shown in Figures 2 - 4 were generated using the master mixes indicated. Amplified products were run on an ABI instrument.

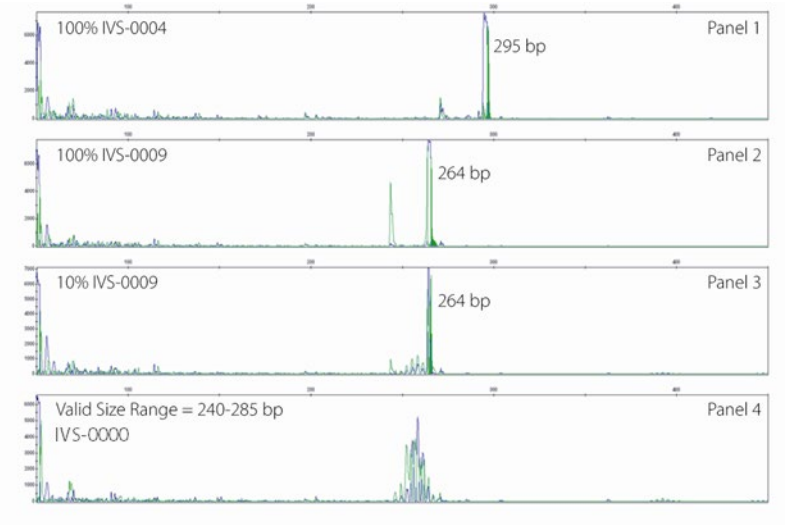


Figure 2. *TCRB* Tube A – 6FAM & HEX

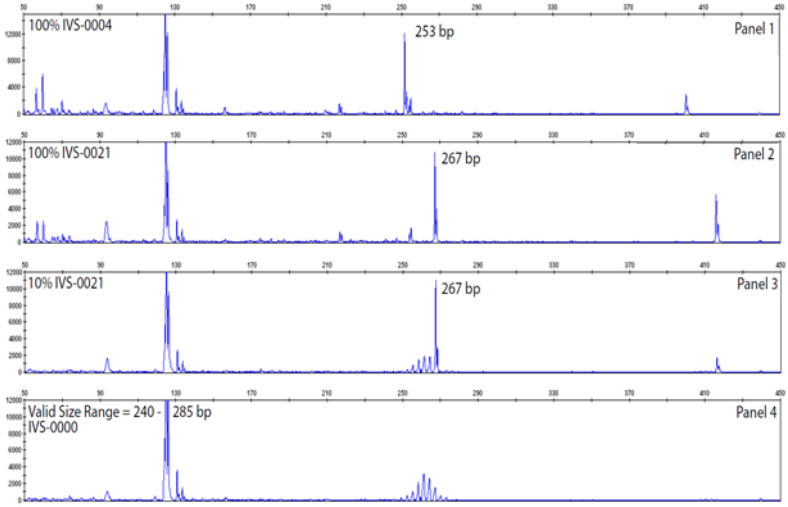


Figure 3. *TCRB* Tube B – 6FAM

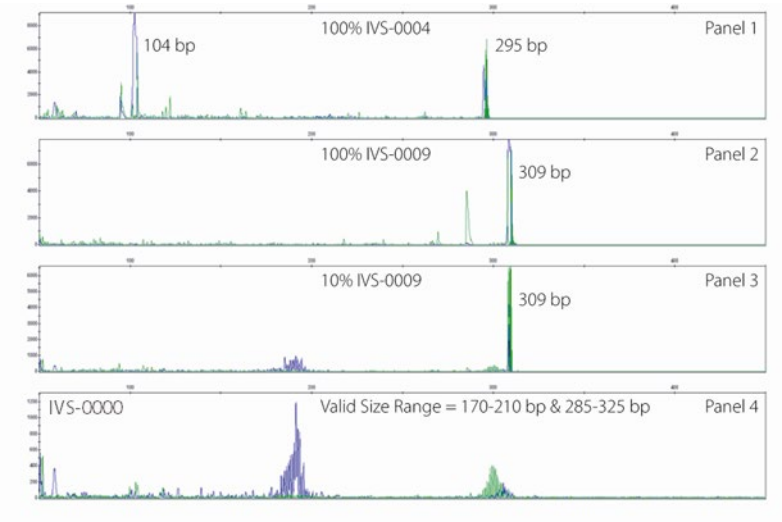


Figure 4. *TCRB* Tube C – 6FAM & HEX

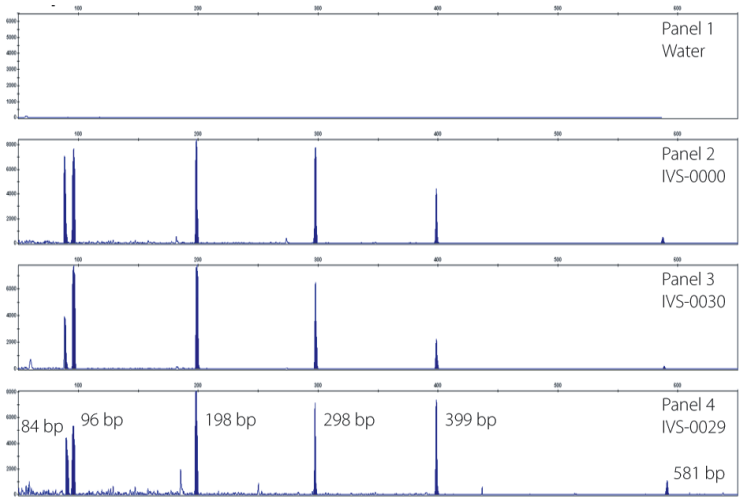


Figure 5. Specimen Control Size Ladder – 6FAM master mix

# 11. Performance Characteristics

This IdentiClone *TCRB* Gene Clonality PCR test is a rapid and reliable procedure that is far more sensitive than Southern Blot (SB) analysis in detecting clonality in suspect lymphoproliferations. The final clinico-histopathological diagnosis correlates well with PCR results in a higher number of patients in comparison with SB results. This is evidenced by two notable papers, one published in 2003 in Leukemia by van Dongen *et al.* and one published in 2005 in the Journal of Molecular Diagnostics (JMD) by Sandberg *et al*.

Table 9. Comparison of PCR and Southern Blot detection

| PCR/SB concordance (*Leukemia*):[2] | | PCR/SB concordance (*JMD*):[3] | |
|---|---|---|---|
| *IGH*: | 93% sensitivity/ 92% specificity | *IGH* + *IGK*: | 85% sensitivity |
| *IGK*: | 90% sensitivity/ 90% specificity | | |
| *IGL*: | 86% sensitivity/ 92% specificity | | |
| *TCRB*: | 86% sensitivity/ 98% specificity | *TCRB*: | 85% sensitivity |
| *TCRG*: | 89% sensitivity/ 94% specificity | | |
| *TCRD*: | 83% sensitivity/ 95% specificity | | |

Table 10. PCR vs. SB analysis relative to histopathology and final diagnosis

| | PCR/SB concordance: | PCR sensitivity: | SB sensitivity |
|---|---|---|---|
| *IGH + IGK*: | 85% | 98% | 39% |
| *TCRB*: | 85% | 96% | 35% |

The study by Sandberg *et al.* was an independent study of 300 patient samples from a variety of sample types. In cases where both PCR and SB analyses were done and results could be correlated with histopathology and a final diagnosis, the diagnostic accuracy of selected IdentiClone tests was determined to be at least 96%. This was far more accurate than SB analysis, which in this study missed 23 clear cases of malignancy and 7 probable malignancies. There were no clear false positive results generated using the IdentiClone tests and there was a high level of precision.[3] In addition, a clear benefit of this assay was that clonal results generated allowed for subsequent detection of patient- and tumor-specific gene rearrangements for minimal residual disease detection.

# 12. Technical and Customer Service

Technical and Customer Service Representatives are available Monday through Friday to answer phone, e-mail or website inquiries.

### Contact Information

Invivoscribe, Inc
10222 Barnes Canyon Road | Building 1 | San Diego | California 92121-2711 | USA
Phone: +1 858 224-6600 | Fax: +1 858 224-6601 | Business Hours: 7:00AM - 5:00 PM PST/PDT
Technical Service: support@invivoscribe.com | Customer Service: sales@invivoscribe.com | Website: www.invivoscribe.com

# 13. References

1. Miller, JE, Wilson, SS, Jaye, DJ, Kronenberg, M. (1999). An automated semiquantitative B and T cell clonality assay. *Molecular Diagnostics.* 4, 101-117.
2. Van Dongen, JJM et al. (2003). Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T cell receptor gene recombinations in suspect lymphoproliferations: Report of the BIOMED-2 Concerted Action BMH4 CT98 3936. *Leukemia*. 17, 2257-2317.
3. Sandberg, Y, et al. (2005). BIOMED-2 multiplex immunoglobulin/T-cell receptor polymerase chain reaction protocols can reliably replace Southern Blot analysis in routine clonality diagnostics. *J. Molecular Diagnostic.* 7, 495-503.
4. van Krieken, JHJM et al. (2007). Improved reliability of lymphoma diagnostics via PCR-based clonality testing: – Report of the BIOMED-2 Concerted Action BHM4-CT98-3936. *Leukemia.* 21, 201-206.

# 14. Symbols

The following symbols are now used in labeling for Invivoscribe diagnostic products.

| | | | |
|---|---|---|---|
| REF | Catalog Number | | Expiration Date |
| VOL | Reagent Volume | EC REP | Authorized Representative in the European Community |
| LOT | Lot Number | | Consult Instructions for Use |
| | Storage Conditions | IVD | For In Vitro Diagnostic Use |
| UDI | Unique Device Identifier | | Manufacturer |
| UK CA | UK Conformity Assessed | UKRP | UK Responsible Person |
| CH REP | Swiss Authorized Representative | CE | European Conformity |

# 15. Legal Notice

## 15.1. Warranty and Liability

Invivoscribe, Inc. (Invivoscribe®) is committed to providing the highest quality products. Invivoscribe® warrants that the products meet or exceed the performance standards described in the Instructions For Use, as to products with such an insert. If a product is covered by product specifications and does not perform as specified, our policy is to replace the product or credit the full purchase price. No other warranties of any kind, expressed or implied, are provided by Invivoscribe®. Invivoscribe® liability shall not exceed the purchase price of the product. Invivoscribe shall have no liability for direct, indirect, consequential or incidental damages arising from the use, results of use, or inability to use its products; product efficacy under purchaser controlled conditions in purchaser's laboratory must be established and continually monitored through purchaser defined and controlled processes including but not limited to testing of positive, negative, and blank controls every time a sample is tested. Ordering, acceptance, and use of product constitutes purchaser acceptance of sole responsibility for assuring product efficacy and purchaser agreement to the limitation of liability set forth in this paragraph.

This product is an *in vitro* diagnostic product is not available for sale or use within North America.

## 15.2. Patents and Trademarks

This product is covered by one or more of the following: European Patent Number 1549764, European Patent Number 2418287, European Patent Number 2460889, Japanese Patent Number 4708029, United States Patent 8859748, and related pending and future applications. All of these patents and applications are licensed exclusively to Invivoscribe®. Additional patents licensed to Invivoscribe covering some of these products apply elsewhere. Many of these products require nucleic acid amplification methods such as Polymerase Chain Reaction (PCR). No license under these patents to use amplification processes or enzymes is conveyed expressly or by implication to the purchaser by the purchase of this product.

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