IdentiClone™

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IdentiClone™ T Cell Receptor Gamma Gene Rearrangement Assay 2.0

CE

For Identification of T Cell Clonality
IVD For *in vitro* Diagnostic Use

Key to Symbols Used

IVD	For *In Vitro* Diagnostic Use
REF	Catalog Number
VOL	Reagent Volume
LOT	Lot Number
	Storage Conditions
	Expiration Date
EC REP	Authorized Representative in the European Community
	Manufacturer
	Consult Instructions for Use

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

invivoscribe

Catalog#	Products	Quantity
REF 9-207-0101	IdentiClone™ T Cell Receptor Gamma Gene Rearrangement Assay 2.0	33 Reactions
REF 9-207-0111	IdentiClone™ T Cell Receptor Gamma Gene Rearrangement Assay 2.0 MegaKit	330 Reactions

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1. Proprietary Name

IdentiClone™ T Cell Receptor Gamma Gene Rearrangement Assay 2.0
IdentiClone™ T Cell Receptor Gamma Gene Rearrangement Assay 2.0 MegaKit

2. Intended Use

The IdentiClone™ T Cell Receptor Gamma Gene Rearrangement Assay 2.0 is an *in vitro* diagnostic product intended for PCR-based detection of clonal T-cell receptor gamma chain gene rearrangements in patients with suspect lymphoproliferations.

Specifically, the T Cell Receptor Gamma Gene Rearrangement Assay 2.0 can be used to identify clonality in suspect lymphoproliferations.

3. Summary and Explanation of the Test

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. 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 gamma chain gene. Amplifying the region with fluorescently labeled primers is followed by fractionation by capillary electrophoresis and analysis by instrument software. This DNA based test is used to detect the vast majority of clonal T-cell populations. Presence or absence of clonality can support the differential diagnosis of reactive lesions and certain T and B cell malignancies.

This assay cannot reliably detect clonality present at less than 5% of the total lymphocyte population. It should be emphasized that the results of molecular clonality testing should always be interpreted in the context of all available clinical, histological and immunophenotypic data.

This test kit consists of a single master mix that contains primers that target the Vγ2, 3, 4, 5, 8, 9, 10, & 11 and Jγ1/Jγ2, JγP, and JγP1/JγP2 regions. The PCR amplicons have an expected size range between 159 and 207 base pairs. The Specimen Control Size Ladder master mix targets multiple genes and generates a series of amplicons of approximately 100, 200, 300, 400, and 600 base pairs to ensure that the quality and quantity of input DNA is adequate to yield a valid result. A single thermocycler program and similar detection methodology is used with all of our Gene Clonality Assays. This improves consistency and facilitates cross training on a broad range of our different assays.

A software based algorithm has been developed for analyzing the peaks that were measured on the capillary electrophoresis instrument. The algorithm calculates the relative peak height ratio (RPR) and a statistical parameter D(x) value for each peak. The RPR is calculated by dividing the height of each peak to the smaller of its neighboring peaks and it must exceed a cutoff of 4.0. The D(x) value is based on a variation of the Kolmogorov-Smirnov test, which compares two empirical distributions and determines whether they are statistically different and its value must be greater than 0.0419.

4. Principles of the Procedure

4.1. Polymerase Chain Reaction (PCR)

PCR assays are routinely used for the identification of clonal T-cell populations. This test amplifies the DNA between primers that target conserved regions within the variable (V) and the joining (J) regions that flank the unique hypervariable antigen-binding region 3 (CDR3). 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 Gaussian distribution (bell-shaped curve) of amplicon products is produced within an expected size range. This Gaussian distribution reflects the heterogeneous population of V-J rearrangements. In certain cases, where lymphocyte DNA is not present, no product is detected. For DNA from samples containing a clonal population, the yield is one or two prominent amplified products (amplicons) within the valid size range.

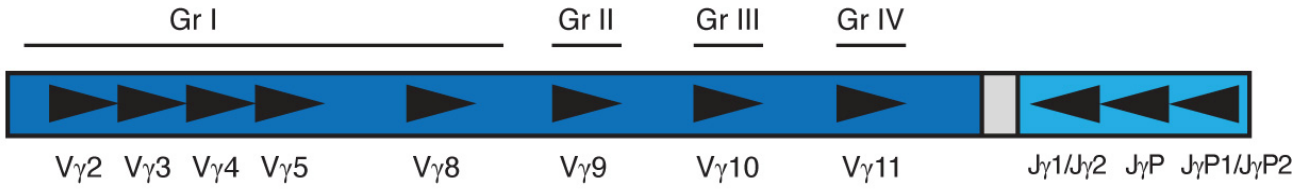


Figure 1. This diagram of the T cell receptor gamma gene shows the approximate placement of the upstream and downstream DNA primers.

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, a multiplex master mix, which targets multiple V and J regions (Figure 1), is required to detect the majority of clonal rearrangements. As indicated, clonal rearrangements are identified as one or two prominent, single-sized products within the background of different-sized amplicon products that form the Gaussian distribution around a statistically favored, average-sized rearrangement.

4.2. Fluorescence Detection

Fluorescence detection is commonly used to resolve the different-sized amplicon products using a capillary electrophoresis instrument. Primers are conjugated with a 6FAM fluorescent dye (fluorophore) so that they can be detected after excitation by a laser in the capillary electrophoresis instrument. This highly sensitive detection system provides single base pair size resolution and relative quantification. Inter and intra-assay reproducibility in size determination using capillary electrophoresis is approximately 1 to 2 base pairs. 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.

5. Reagents

5.1. Reagent Components

REF	9-207-0101	IdentiClone™ T Cell Receptor Gamma Gene Rearrangement Assay 2.0	33 Reactions
REF	9-207-0111	IdentiClone™ T Cell Receptor Gamma Gene Rearrangement Assay 2.0 MegaKit	330 Reactions

Table 1

Reagent	Catalog #	Reagent Components (active ingredients)	Unit Quantity	9-207-0101 # of Units	9-207-0111 # of Units	Storage Temp.
Master Mixes	2-207-0091	**TCRG – 6FAM** Multiple oligonucleotides targeting the Vγ2, 3, 4, 5, 8, 9, 10, & 11 and Jγ1/Jγ2, JγP, and JγP1/JγP2 regions of the T cell receptor gamma gene in a buffered salt solution.	1500 µL	1	10	-65 °C to -85 °C
Template Amplification Control Master Mix	2-096-0021	**Specimen Control Size Ladder – 6FAM** Multiple oligonucleotides targeting housekeeping genes.	1500 µL	1	10	-65 °C to -85 °C
Positive Control DNA	4-088-3320	**5% TCRG Positive Control DNA** 50 µg/mL of DNA in 1/10th TE solution	50 µL	1	5	2 °C to 8 °C or -65 °C to -85 °C
Negative (Normal) Control DNA	4-092-0020	**TCRG Negative Control DNA** 50 µg/mL of DNA in 1/10th TE solution	50 µL	1	5	2 °C to 8 °C or -65 °C to -85 °C

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

5.2. Warnings and Precautions

1. IVD **This Product is for *in vitro* Diagnostic Use**
2. The assay kit should be used as a system. Do not substitute other manufacturer's reagents. Dilution, reduction of 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.

3. Materials are stable until the labeled expiration date when stored and handled as directed. Do not use kits beyond their expiration date.
4. Close adherence to the protocol will assure optimal performance and reproducibility. Care should be taken to ensure use of correct thermocycler program, as other programs may provide inaccurate/faulty data, such as false positive and false negative results.
5. Do not mix or combine reagents from kits with different lot numbers.
6. Laboratory personnel are reminded to wear appropriate personal protective equipment and follow good laboratory practices and universal precautions when working with specimens. Specimens should be handled in approved biological safety containment facilities and opened only in certified biological safety cabinets. It is recommended that glass distilled de-ionized molecular biology grade water be used with the preparation of specimen DNA.
7. Due to the high analytical sensitivity of this test, extreme care should be taken to avoid the contamination of reagents or amplification mixtures with samples, controls or amplified materials. All reagents should be closely monitored for signs of contamination (*e.g.*, negative controls giving positive signals). Discard reagents suspected of contamination.
8. To minimize contamination, wear clean gloves when handling samples and reagents and routinely clean work areas and pipettes prior to doing PCR.
9. Autoclaving does not eliminate DNA contamination. Work flow in the PCR laboratory should always be in a one way direction between separate work areas; beginning in Master Mix Preparation, moving 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.
10. All pipettes, pipette tips, and any equipment used in a particular area should be dedicated to and kept to that area of the laboratory.
11. Sterile, disposable plastic ware should be used whenever possible to avoid RNase, DNase, or cross-contamination.

5.3. Storage and Handling

- For any duration other than immediate use, **assay kits should be stored at -65 °C to -85 °C**.
- The optimum storage temperature for the DNA controls is 2 °C to 8 °C, but DNA controls can also be stored at -65 °C to -85 °C.
- All reagents and controls must be thawed and vortexed or mixed thoroughly prior to use to ensure that they are mixed completely. Excessive vortexing may cause labeled primers to lose their fluorophores.
- Materials are stable until the labeled expiration date when stored and handled as directed. Do not use kits beyond their expiration date.
- The PCR master mixes and controls have been validated for 6 freeze-thaw cycles with no loss in performance. Aliquot reagents into sterile o-ring screw-cap tubes if more freeze-thaw cycles are necessary.

6. Instruments

6.1. Thermocycler

- Use or Function: Amplification of DNA samples
- 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 *8.4 Amplification* for thermocycler program.

6.2. ABI Capillary Electrophoresis Instruments

- Use or Function: Fragment detection and analysis
- Performance Characteristics and Specification:
  The following capillary electrophoresis instruments will meet the performance needs for this assay:
  - 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 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 *8.2 Materials Required But Not Provided.*
- Use the default settings for your polymer and capillary type.

- See section *8.5 ABI Fluorescence Detection* for sample preparation.

7. Specimen Collection and Preparation

7.1. Precautions

Biological specimens from humans may contain potentially infectious materials. All specimens should be handled in accordance with the OSHA Standard on Bloodborne Pathogens or Biosafety Level 2.

7.2. Interfering Substances

The following substances are known to interfere with PCR:

- Divalent cation chelators
- Low retention pipette tips
- EDTA
- Heparin

7.3. Specimen Requirements and Handling

This assay tests genomic DNA extracted and purified from peripheral blood, bone marrow aspirates or paraffin embedded tissue.

7.4. Sample Preparation

Extract the genomic DNA from patient specimens as soon as possible. Resuspend DNA to a final concentration of 10 µg to 200 µg per ml in $1/10^{th}$ 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.

7.5. Sample Storage

Genomic DNA should be stored at 2 °C to 8 °C or at -65 °C to -85 °C until use.

8. Assay Procedure

8.1. Materials Provided

Table 2

Catalog #	Description
2-207-0091	TCRG – 6FAM
2-096-0021	Specimen Control Size Ladder – 6FAM
4-088-3320	5% TCRG Positive Control DNA
4-092-0020	TCRG Negative Control DNA

8.2. Materials Required But Not Provided

Table 3

Reagent/Material	Recommended Reagents/Materials and Suppliers	Notes
DNA Polymerase	Applied Biosystems: AmpliTaq Gold® DNA Polymerase (Cat# N808-0241)	N/A
Glass Distilled De-ionized Molecular Biology Grade or USP Water	N/A	Water must be sterile and free of DNase and RNase.
Calibrated Pipettes	N/A	Must be able to accurately measure volumes between 1µl and 1000µl.
Thermocycler	N/A	N/A
Vortex Mixer	N/A	N/A
PCR plates or tubes	N/A	Sterile
Filter barrier pipette tips	N/A	Sterile, RNase/DNase/Pyrogen-free
Microcentrifuge tubes	N/A	Sterile

Reagent/Material	Recommended Reagents/Materials and Suppliers	Notes
ABI Capillary Electrophoresis Instrument	Applied Biosystems: ABI 3100, 3130, or 3500 series	N/A
Hi-Di Formamide	Invivoscribe Technologies: HI-Deionized Formamide (Cat# 6-098-0041) Applied Biosystems: Hi-Di™ Formamide (Cat# 4311320)	N/A
Size Standards	Invivoscribe Technologies: Hi-Di Formamide w/ROX size standards for ABI 3100 (Cat# 6-098-0061) Applied Biosystems: For ABI 3100 or 3130 instruments: GeneScan™ - 400HD [ROX] ™ (Cat# 402985) For ABI 3500 instruments: GeneScan™ - 600 [LIZ] ™ v2.0 (Cat# 4408399)	N/A
Spectral Calibration Dye Sets	Applied Biosystems: For ABI 3100 and 3130 instruments: DS-30 Matrix Standard Kit (Dye Set D) (Cat# 4345827) For ABI 3500 instruments: DS-33 Matrix Standard Kit (Dye Set G5) (Cat# 4345833)	Dye set used to spectrally calibrate ABI instrument for use with 6FAM, HEX, NED, and ROX
Polymer	Applied Biosystems: POP-7 Polymer: POP-7™ for 3130/3130XL/3500XL Genetic Analyzers (Cat# 4352759)	N/A

8.3. Reagent Preparation

- All samples should be tested using the Specimen Control Size Ladder master mix. This is to ensure that no inhibitors of amplification are present and there is DNA of sufficient quality and quantity to generate a valid result.
- All samples must be tested in duplicate. If duplicate testing provides inconsistent results, re-testing or re-evaluation of the sample is necessary.
- **Positive**, **negative,** and **no template** controls must be tested.

1. Using gloved hands, remove the master mixes from the freezer. Allow the tubes to thaw completely; then gently vortex to mix.
2. Remove the calculated volume of each master mix to individual microcentrifuge tubes. The aliquot volume is 45 µl for each reaction. We recommend adding an additional reaction for every 15 reactions to ensure an adequate volume is available. For the TCRG – 6FAM master mix, the number of reactions (**n**) should be:

n = 2 × # of samples	(Run each sample in duplicate)
+ 1	5% TCRG Positive Control DNA
+ 1	TCRG Negative Control DNA
+ 1	No Template Control (water)
+ 1	Additional Reaction
n = 2 × # of samples + 4	**Total**

The total aliquot volume for the TCRG – 6FAM master mix will be **n** × 45µl.
For the Specimen Control Size Ladder master mix, the number of reactions (**m**) will be:

m = # of samples	(Run each sample in singlicate)
+ 1	TCRG Negative Control DNA
+ 1	No Template Control (water)
+ 1	Additional Reaction
m = # of samples + 3	**Total**

The total aliquot volume for the Specimen Control Size Ladder master mix will be **m** × 45µl.

3. Add 1.25 units (or 0.25µl at 5 units/µl) of AmpliTaq Gold DNA polymerase per reaction to each master mix. The total AmpliTaq Gold DNA polymerase added to the TCRG master mix will be **n** × 0.25µl, and **m** × 0.25µl for the Specimen Control Size Ladder master mix. Gently vortex to mix.
4. For each reaction, aliquot 45µl of the appropriate master mix + DNA polymerase solution into individual wells in a PCR plate or tube.

5. Add 5µl 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.
6. Cap or cover the PCR plate.
7. Samples are now ready to be amplified on a thermocycler.

Quick Guide:

For each master mix and **n** reactions, mix:

n × 45 µl	Master Mix
n × 0.25 µl	AmpliTaq Gold DNA polymerase

Vortex gently to mix.
Aliquot **45µl** of master mix + DNA polymerase solution into each reaction well.
Add **5µl** of appropriate sample to each well.
Total reaction volume = **50µl**

8.4. Amplification

1. Amplify the samples using the following PCR program:
(**Note**: We recommend using the **calculated** option for temperature measurement with the BioRad MJ Research PTC thermocyclers.)

PCR Program
Step 1: 95 °C for 7 minutes
Step 2: 95 °C for 45 seconds
Step 3: 60 °C for 45 seconds
Step 4: 72 °C for 90 seconds
Step 5: Go to step 2; 34 more times
Step 6: 72 °C for 10 minutes
Step 7: 15 °C forever

2. Remove the amplification plate or tubes from the thermocycler.

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

8.5. ABI Fluorescence Detection

- Please note that for ABI fluorescence detection a preceding peak is often seen and is an artifact due to the detection method the ABI platforms use. Preceding peaks are sometimes skewed and have bases that slope on the right side towards the real peak. This is especially evident in the Specimen Control Size Ladder master mix where the 96-base pair peak has a preceding peak that shows up at 84 base pairs.

ABI 3100 and 3130 Platforms:

1. In a new microcentrifuge tube, mix an appropriate amount (10 µl per PCR reaction) of Hi-Di Formamide with ROX Size Standards[a]. Vortex well.
2. In a new 96-well PCR plate, add 10 µl of Hi-Di Formamide with ROX size standards to individual wells for each PCR reaction.
3. Transfer 1 µl of each PCR reaction to the wells containing Hi-Di Formamide with ROX size standards. Add only one sample per well. Pipette up and down to mix.
4. Cap or cover the PCR plate.
5. Heat denature the samples at 95 ºC for 2 minutes, then snap chill on ice for 5 minutes.
6. Prepare a **sample sheet** and **injection list** for the samples.
7. Run the samples on an ABI 3100/3130 capillary electrophoresis instrument according to its user manual[b].
8. Data are automatically displayed as size and color specific peaks. Review profile and controls, report results. (See sections 9 *Interpretation of Results* and *11 Expected Values* below.)

ABI 3500 Platforms:

1. In a new microcentrifuge tube, mix an appropriate amount (9.5 µl per PCR reaction) of Hi-Di Formamide with LIZ Size Standards[a]. Vortex well.
2. In a new 96-well PCR plate, add 9.5 µl of Hi-Di Formamide with LIZ size standards to individual wells for each PCR reaction.
3. Transfer 0.5 µl of each PCR reaction to the wells containing Hi-Di Formamide with LIZ size standards. Add only one sample per well. Pipette up and down to mix.
4. Cap or cover the PCR plate.
5. Heat denature the samples at 95 ºC for 3 minutes, then snap chill on ice for 5 minutes.
6. Prepare a sample sheet and injection list for the samples.
7. Run the samples on an ABI 3500 capillary electrophoresis instrument according to its user manual.
8. Data are automatically displayed as size and color specific peaks. Review profile and controls, report results. (See sections 9 *Interpretation of Results* and *11 Expected Values*)

Note [a]: Please see Applied Biosystems' accompanying product insert for mixing Hi-Di Formamide with size standards for different ABI instruments.
Note [b]: As the samples are run on the machine, they are fractionated, detected and analyzed by the instrument. Runs are 20-24 minutes in duration. The ABI capillary electrophoresis instruments routinely handle 2 runs per hour (for the 16- and 24-capillary instruments this is equal to 768 and 1152 samples per day, respectively), and automatically analyze and store data for review or comparison with other test results.

8.6. Data Analysis

The TCRG Algorithm worksheet has been developed to analyze the TCRG V2 data output.

1. Open the TCRG Algorithm Worksheet (the worksheet requires Microsoft Excel).
2. Add raw data files derived from CE analysis to a new project in GeneMapper software.
3. Verify that Analysis Method selected is "Microsatellite Default" and that the appropriate Size Standard is selected.
4. It may be necessary to lower the Minimum Peak Height threshold in order to detect all peaks in a Gaussian distribution. In order to do this, select GeneMapper Manager from the Tools Menu, go to the Analysis Methods tab, open the "Microsatellite Default" Analysis Method Editor. Go to the Peak Detector tab, select User Specified (rfu) toggle, and input the desired peak height for the Blue dye.
5. In the Analysis menu, select Analyze.
6. For each analyzed sample file, open the associated display plot.
7. To ensure that only the Blue Dye is shown in the display plot, go to the View menu, select Dyes → Blue Dye.
8. Next, in the View menu, choose Tables → Sizing Table.
9. Highlight the display plot peaks in the valid size range from 159 bp to 207 bp.
10. In the Sizing Table, Copy the Size (bp) and Height (RFU) column data for the highlighted peaks within the valid size range.
11. Paste the peak size and height data into the unlocked portion of the TCRG Algorithm worksheet (cells are highlighted in grey). Note: for GeneMapper version 3.5 and lower, this data must be entered into the worksheet manually.
12. The worksheet will output a summary of RPR, D(x), and %RFU(max) for the five peaks that are the most significant outliers from a normal Gaussian distribution.
13. If a peak in the summary table meets the criteria for a clonal peak as defined in the TCRG Algorithm worksheet, it will read "Yes" in the column titled "Significant?."
14. If a peak in the summary table does not meet the criteria for a clonal peak as defined in the TCRG Algorithm worksheet, it will read "No" in the column titled "Significant?."
15. The following criteria are implemented in the worksheet to define peaks as Positive for Clonality:
    - The D(x) value of the suspected clonal peak, as calculated within the locked portion of the worksheet, must be ≥ 0.0419.
    - The RPR of the suspected clonal peak, compared to the smallest adjacent peak, must be ≥ 4X.
    - The RFU of the suspected clonal peak must be ≥ 20% of the RFU of the highest peak in that sample.
    - If a peak is present + 1 bp from a clonal peak, it can be ignored if RFU (Pk + 1) ≤ RFU (Pk).
    - There must be ≥ 2 bp difference between two positive peaks.

- Samples must be run in duplicate, and both replicates must show positive results for a suspected peak.
- The size of the suspected clonal peaks in both replicates must be within ± 1 bp from each other.

8.7. Quality Control

Positive and negative controls are provided with the kit and must be included with each assay run. In addition, a no template control (e.g. water) must also be included. 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 under section *11.1 Expected Size of Amplified Products*. Additional controls and sensitivity controls (dilutions of positive controls into our negative control) are available from Invivoscribe Technologies.

8.8. Assay Control

The amplicon sizes listed in Table 4 were determined using an ABI 3130 platform. The amplicon sizes measured on your specific capillary electrophoresis instrument may differ by 1 to 4 base pairs 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. **Note**: "Color" indicates the color of products generated with the master mix when using the default color assignment on ABI fluorescence detection systems.

Table 4

Master Mix	Target	Color	Control DNA	Cat#	Product Size in Base Pairs	Expected Algorithm Results
TCRG - 6FAM	Vγ1-Vγ11 + Jγ1/Jγ2, JγP, JγP1/JγP2	**Blue**	**Specified Size Range** 5% TCRG Positive Control DNA	--- 4-088-3320	**159-207** 194, 196	**One peak at 196 bp flagged as "Significant"**
Specimen Control Size Ladder	Multiple Genes	**Blue**	TCRG Negative Control DNA	4-092-0020	84, 96, 200, 300, 400, 600[a]:	**N/A**

Note [a]: Because smaller PCR fragments are preferentially amplified, it is not unusual for the 600 bp fragment to have a diminished signal or to be missing entirely. For ABI fluorescence detection, the 600 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.

9. Interpretation of Results

Although positive results are highly suggestive of malignancy, both positive and negative results should be interpreted in the context of all clinical information and laboratory test results. The size range for the TCRG – 6FAM master mix has been determined to be 159 bp to 207 bp by testing positive and negative control samples. However, valid clonal TCR gamma rearrangements can occur outside the specified size range. Product(s) that are suspect TCR gamma gene rearrangement(s) that lie outside the specified size range should be sequenced to confirm their identity.

9.1. Analysis

1. Samples that fail to amplify following repeat testing should be reported as "**A result cannot be reported on this specimen because the DNA was of insufficient quantity or quality for analysis**".
2. If the positive or negative control reactions fail, testing must be repeated.
3. If samples run in duplicate yield differing results, the samples should be re-tested and/or re-evaluated for sample switching.
4. **All assay controls must be examined prior to interpretation of sample results. If the controls do not yield the correct results, the assay is not valid and the samples must not be interpreted**.

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

Table 5

Type of Control	Expected Result	Aberrant Result
No Template Control	No amplification present: continue with analysis	Amplification present: check for contamination and repeat the assay.
TCRG Negative Control	Normal Gaussian distribution from 159 bp to 207 bp. No clonal peaks are flagged by Algorithm worksheet. Continue with analysis.	Algorithm worksheet flags one or more peaks as "Significant": Repeat the assay

Type of Control	Expected Result	Aberrant Result
5% TCRG Positive Control (This can also be an extraction control if positive control material is taken through extraction processes)	Peaks present at 194 bp, 196 bp. Algorithm worksheet flags at least the196 bp as "Significant." The 194 bp peak may or may not be identified as "Significant". Continue with analysis.	Algorithm worksheet does not flag peak at 196 bp as "Significant": Repeat the assay.
Specimen Control Size Ladder	If the 100, 200, 300, and 400 bp peaks are seen, continue with analysis. Because smaller PCR fragments are preferentially amplified, it is not unusual for the 600 bp fragment to have a diminished signal or to be missing entirely.	If no peaks are detected, repeat the assay unless specimen tests positive. If only the 100, 200, or 300 bp peaks are present, re-evaluate sample for DNA degradation unless specimen tests positive.

9.2. Sample Interpretation

Given that the controls produce expected results, the clinical samples should be interpreted as follows:

- One or two significant peaks flagged by the Algorithm worksheet within the valid size range are reported as:

  "**Positive for the detection of clonal T cell receptor gamma chain gene rearrangement(s) 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**."

- Three or more significant peaks flagged by the Algorithm worksheet within the valid size range are reported as:

  "**T cell receptor gamma chain gene rearrangements are consistent with the detection of biclonality or oligoclonality**."

- An absence of significant peaks flagged by the Algorithm worksheet within the valid size range is reported as:

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

  Note: A visual confirmation should be conducted; the electropherogram should be visually reviewed to ensure that the algorithm interpreted the pattern correctly.

10. Limitations of Procedure

- This assay does not identify 100% of clonal cell populations.
- This assay cannot reliably detect below 5 positive cells per 100 total cells.
- The results of molecular clonality tests should always be interpreted in the context of clinical, histological and immunophenotypic data.
- The algorithm requires a reasonably consistent signal background and that the data is entered correctly. Gaps in the background can cause the algorithm to call a sample incorrectly. All electropherograms should be reviewed to confirm the validity of the interpretation.
- PCR-based assays are subject to interference by degradation of DNA or to inhibition of PCR due to EDTA, heparin, or other agents.

11. Expected Values

11.1. Expected Size of Amplified Products

- The amplicon sizes listed were determined using an ABI 3130 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 6

Master Mix	Target	Color	Control DNA	Cat#	Product Size in Base Pairs	Expected Algorithm Results
			Specified Size Range	---	**159-207**	
TCRG-6FAM	All V and J genes	**Blue**	TCRG Negative Control DNA	4-092-0020	159-207	No Significant Peaks
	Vγ9, Vγ10 + Jγ1/Jγ2	**Blue**	5% TCRG Positive Control DNA	4-088-3320	194 & 196	Significant Peak at 196 bp and possibly second peak at 194 bp
Specimen Control Size Ladder	Multiple Genes	**Blue**	Any Human DNA	---	84, 96, 200, 300, 400, 600 [a]	N/A

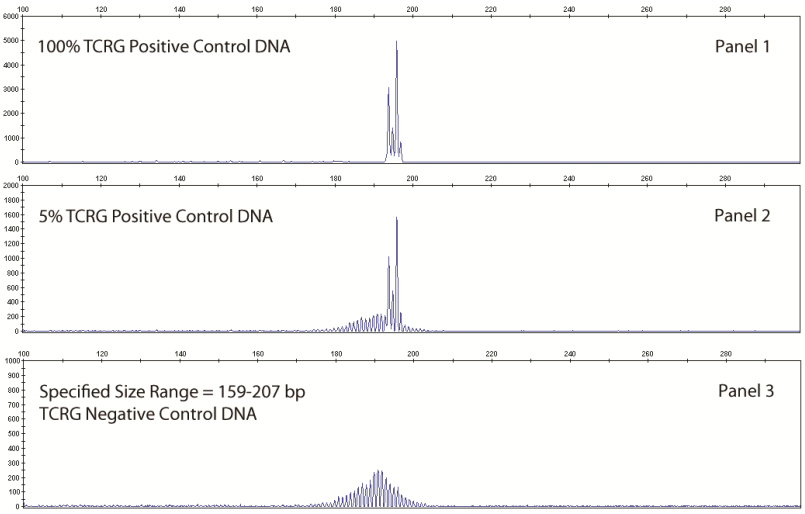
Note [a]: Because smaller PCR fragments are preferentially amplified, it is not unusual for the 600nt fragment to have a diminished signal or to be missing entirely. For ABI fluorescence detection the 600 bp peak may not appear during normal run times. In addition, the size of this peak may differ by over 30 bp when fragment size is extrapolated using the GeneScan - 400HD [ROX] size standards.

11.2. Sample Data

The data shown below was generated using the TCRG-6FAM Master Mix. Amplified products were run on an ABI 3130 instrument.

- Panel 1 displays data generated testing the 100% TCRG Positive Control DNA.
- Panel 2 displays data generated testing the 5% TCRG Positive Control DNA.
- Panel 3 displays data generated testing the TCRG Negative Control DNA.

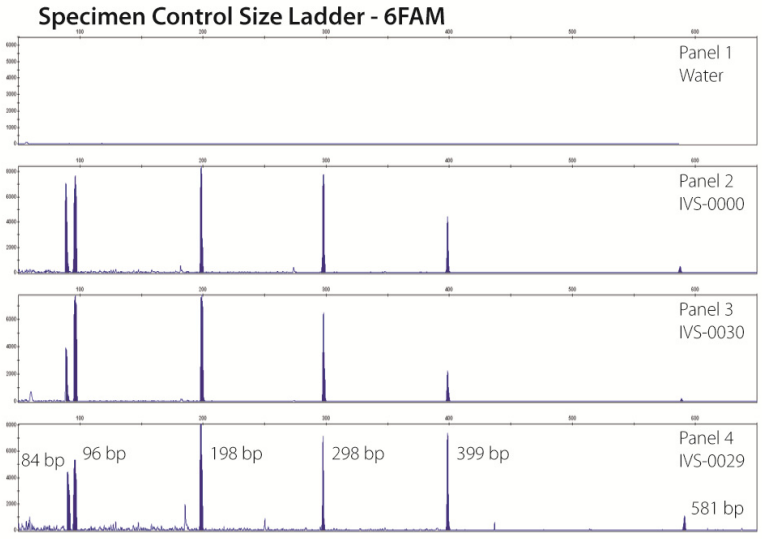
Figure 2



For the Specimen Control Size Ladder master mix:

- Panel 1 displays data generated testing a negative water control.
- Panel 2 displays data generated testing the IVS-0000 Polyclonal Control DNA.
- Panels 3 and 4 display data generated testing two different 100% clonal control DNAs.

Figure 3



12. Performance Characteristics

The assay was able to detect clonal rearrangements in 11 positive control cell lines. The following well-characterized T cell leukemia cell lines known to be positive for TCRG rearrangements were tested with TCRG – 6FAM master mix, and the results are shown in Table 7. Two prominent peaks were detected with each of the cell lines.

Table 7

Cell Line	IVS Part Number	Peak One (bp)	Peak Two (bp)
100% IVS-0004	4-088-0190	178.8	187.9
100% IVS-0005	4-088-0250	173.0	198.3
100% IVS-0008	4-088-0430	195.1	206.7
100% IVS-0009	4-088-0490	187.8	190.5
100% IVS-0016	4-088-0910	169.4	193.7
100% IVS-0021	4-088-1210	183.1	188.0
100% IVS-0039	4-088-2290	193.9	195.9
DND-41	N/A	168.9	188.8
PF-382	N/A	190.6	200.1
MOLT-13	N/A	187.9	190.6

The assay showed robust results (Table 8) when tested with IVS-0039 DNA (200 ng/µl) diluted into tonsil DNA (200 ng/µl) at 5%, 10%, 25%, 50% and 75% (v/v).

Table 8

		Peak One				Peak Two			
		Product Size (bp)	**D(x) Value**	**RPR Ratio**	**Significant?**	**Product Size (bp)**	**D(x) Value**	**RPR Ratio**	**Significant?**
5% IVS-0039	rep 1	196.20	0.2330	6.04	Yes	194.16	0.1208	2.93	No
	rep 2	195.81	0.1803	5.32	Yes	193.75	0.0993	2.65	No
	rep 3	195.71	0.1872	6.68	Yes	193.65	0.0962	2.45	No
	rep 4	196.18	0.2080	6.34	Yes	194.09	0.0941	2.88	No
	rep 5	195.79	0.1833	6.07	Yes	193.77	0.0931	2.70	No
10% IVS-0039	rep 1	196.24	0.3573	9.76	Yes	194.31	0.2115	5.15	Yes
	rep 2	196.15	0.3382	9.18	Yes	193.95	0.1877	4.03	Yes
	rep 3	195.66	0.2790	8.32	Yes	193.62	0.1819	4.24	Yes
	rep 4	196.15	0.3382	9.18	Yes	193.95	0.1877	4.03	Yes
	rep 5	195.80	0.2983	7.77	Yes	193.75	0.1830	4.69	Yes
25% IVS-0039	rep 1	196.02	0.3947	8.00	Yes	194.16	0.3730	22.2	Yes
	rep 2	196.11	0.3216	6.07	Yes	194.17	0.3568	20.09	Yes
	rep 3	195.72	0.4059	9.39	Yes	193.66	0.3451	17.02	Yes
	rep 4	196.11	0.3212	6.07	Yes	194.17	0.3561	20.09	Yes
	rep 5	195.71	0.4316	10.24	Yes	193.65	0.3482	17.09	Yes
50% IVS-0039	rep 1	196.15	0.2939	4.58	Yes	194.23	0.4545	630.3	Yes
	rep 2	195.67	0.3817	7.31	Yes	193.69	0.4686	120.4	Yes
	rep 3	195.66	0.4503	9.17	Yes	193.62	0.4672	28.15	Yes
	rep 4	196.07	0.3586	5.73	Yes	194.11	0.4607	32.59	Yes
	rep 5	195.67	0.4404	8.85	Yes	193.69	0.4626	124.4	Yes
75% IVS-0039	rep 1	196.11	0.2387	3.36	No	193.89	0.3532	58.40	Yes
	rep 2	195.72	0.3154	5.29	Yes	193.66	0.4799	71.81	Yes
	rep 3	195.62	0.4520	9.16	Yes	193.57	0.4811	110.9	Yes
	rep 4	196.15	0.2911	4.45	Yes	194.14	0.4387	79.15	Yes
	rep 5	195.71	0.3301	5.86	Yes	193.65	0.4708	78.47	Yes

The assay, when performed in combination with the TCRG Algorithm worksheet, was capable of detecting DNA from 6 control cell lines (200 ng/µl) diluted into tonsil DNA (200 ng/µl) at 5% (v/v) and the results are shown in Table 9.

Table 9

		Peak One				Peak Two			
		Product Size (bp)	**D(x)**	**RPR Ratio**	**Significant?**	**Product Size (bp)**	**D(x)**	**RPR Ratio**	**Significant?**
5% IVS-0004	rep 1	178.87	0.2964	42.50	Yes	184.69	0.1322	5.61	Yes
	rep 2	184.36	0.1193	27.59	Yes	178.39	0.0908	21.13	Yes
	rep 3	184.32	0.1266	22.96	Yes	178.32	0.1041	23.93	Yes
	rep 4	184.69	0.1200	14.97	Yes	178.78	0.1009	29.82	Yes
	rep 5	184.36	0.1342	12.12	Yes	178.40	0.1146	32.07	Yes
5% IVS-0016	rep 1	169.38	0.1035	115.9	Yes	193.71	0.1016	2.32	No
	rep 2	169.03	0.0918	159.0	Yes	193.50	0.0857	2.24	No
	rep 3	168.99	0.0975	55.00	Yes	193.41	0.0791	2.25	No
	rep 4	169.38	0.1028	100.9	Yes	193.77	0.1041	2.45	No
	rep 5	169.00	0.0957	55.0	Yes	193.53	0.0944	2.50	No
5% IVS-0021	rep 1	187.91	0.1120	7.28	Yes	182.92	0.1239	14.57	Yes
	rep 2	187.58	0.1003	5.67	Yes	182.53	0.1298	14.23	Yes
	rep 3	182.50	0.0950	35.5	Yes	187.50	0.1110	5.54	Yes
	rep 4	187.92	0.1112	6.99	Yes	183.01	0.1238	12.68	Yes
	rep 5	187.62	0.0978	6.09	Yes	182.67	0.1253	24.69	Yes
5% IVS-0039	rep 1	195.97	0.2907	8.46	Yes	193.95	0.1576	3.60	No
	rep 2	195.70	0.2221	6.74	Yes	193.59	0.1321	3.04	No
	rep 3	195.56	0.2010	6.86	Yes	193.53	0.1244	2.84	No
	rep 4	196.01	0.2942	8.05	Yes	194.01	0.1484	3.18	No
	rep 5	195.71	0.2513	7.53	Yes	193.65	0.1470	3.36	No
5% PF-382	rep 1	191.84	0.2784	5.77	Yes	158.41	0.3057	123.5	Yes
	rep 2	191.48	0.2558	5.30	Yes	158.24	0.2739	125.7	Yes
	rep 3	191.57	0.2418	5.44	Yes	158.15	0.2787	115.1	Yes
	rep 4	191.84	0.2822	5.68	Yes	158.33	0.2811	118.8	Yes
	rep 5	191.55	0.2524	5.63	Yes	158.15	0.2883	93.0	Yes
5% MOLT-13	rep 1	190.74	0.2147	3.97	No	187.92	0.1292	6.96	Yes
	rep 2	190.46	0.1806	3.51	No	187.60	0.1081	4.96	Yes
	rep 3	190.46	0.1731	3.42	No	187.53	0.1039	5.27	Yes
	rep 4	190.64	0.2132	4.10	Yes	187.93	0.1215	5.69	Yes
	rep 5	190.46	0.1983	6.19	Yes	187.57	0.1114	5.80	Yes

Note: 5% IVS-0004 is P/N 4-088-0230, 5% IVS-0016 is P/N 4-088-0950, 5% IVS-0021 is P/N 4-088-1250 and 5% IVS-0039 is P/N 4-088-2330

Using clinical samples, the TCRG V2 assay results were compared to Roche 454 sequencing for the identification T-cell receptor gamma gene rearrangements. For the 454 sequencing, any DNA sequence that was present at levels greater than 5% of the total sequences detected was considered a clonal event. If more than 2 sequences exceeded the 5% threshold, that sample was defined as oligoclonal. The TCRG V2 assay had 100% concordance for the 7 samples that were identified

as clonal by sequencing. There was 75% concordance for the 12 samples that were either negative for a clonal event or were oligoclonal. Sample types included peripheral blood, bone marrow, and FFPE samples. It is important to note that the presence or absence of clonal peaks in a clinical sample does not always correlate with actual clinical outcomes.

13. Bibliography

1. Miller, JE*, et al*., An automated semiquantitative B and T cell clonality assay. *Mol. Diag*. 1999, **4(2)**:101-117.

14. Technical and Customer Service

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