Identicleone™ IGH + IGK
B-Cell Clonality Assay

For Identification of Clonal Immunoglobulin Heavy Chain and Kappa Light Chain Gene Rearrangements

For In Vitro Diagnostic Use

Key to Symbols Used

IVO For In Vitro Diagnostic Use

REF Catalog Number

VOL Reagent Volume

LOT Lot Number

Storage Conditions

Expiration Date

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)

<table>
<thead>
<tr>
<th>Catalog#</th>
<th>Products</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>REF 9-100-0031</td>
<td>IdentiClone™ IGH + IGK B-Cell Clonality Assay – ABI Fluorescence Detection</td>
<td>33 Reactions</td>
</tr>
<tr>
<td>REF 9-100-0041</td>
<td>IdentiClone™ IGH + IGK B-Cell Clonality Assay MegaKit – ABI Fluorescence Detection</td>
<td>330 Reactions</td>
</tr>
</tbody>
</table>
1. Proprietary Name

IdentiClone™ IGH + IGK B-Cell Clonality Assay – ABI Fluorescence Detection
IdentiClone™ IGH + IGK B-Cell Clonality Assay MegaKit – ABI Fluorescence Detection

2. Intended Use

The IdentiClone™ IGH + IGK B-Cell Clonality Assay is an *in vitro* diagnostic product intended for PCR-based detection of clonal immunoglobulin heavy chain and kappa light chain gene rearrangements in patients with suspect lymphoproliferations.

Specifically, the IGH + IGK B-Cell Clonality Assay can be used to:
- Identify clonality in atypical lymphoproliferative disorders
- Support a differential diagnosis between reactive lesions and hematologic malignancies
- Assign presumptive lineage in mature monoclonal lymphoproliferative disorders
- Identify tumor-specific markers (IGH and IGK gene rearrangements) for post-treatment monitoring
- Monitor and evaluate disease recurrence

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 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. This IdentiClone™ PCR assay employs multiple consensus DNA primers that target conserved genetic regions within the immunoglobulin heavy chain and kappa light chain genes. This test is used to detect the vast majority of clonal B-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.

*InVivoScribe Technologies’* 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. In a *Leukemia* 2007 article, testing for both IGH and IGK gene rearrangements led to 99% sensitivity, compared to 88% for IGH and 88% for IGK when tested alone. It may also increase the reliability of the tests as it’s more likely that the clonal products will be detected in more than one tube.

The ABI detection based assays cannot reliably detect clonal populations comprising less than 1% of the total lymphocyte cell population. It should be emphasized that the results of molecular clonality tests should always be interpreted in the context of clinical, histological and immunophenotypic data.

This test kit includes 6 master mixes. The IGH Tube A, B, and C master mixes target the framework 1, 2, and 3 regions (respectively) within the variable region, and the joining region of the immunoglobulin heavy chain locus. The IGK Tube A master mix targets the variable (V) and the joining (J) regions of the Ig kappa light chain locus. Whereas the IGK Tube B master mix targets kappa deleting element (Kde) rearrangements with the variable (V) region and the intragenic Jκ-Cκ region. The resulting Vκ-Kde and Jκ-Cκ intron-Kde rearrangements are a result of unsuccessful rearrangements retained by the B-cell. Lastly, the Specimen Control Size Ladder master mix, targets multiple genes and generates a series of amplicons of approximately 100, 200, 300, 400, and 600 basepairs to ensure that the quality and quantity of input DNA is adequate to yield a valid result. A single thermocycler 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.
4. Principles of the Procedure

4.1. Polymerase Chain Reaction (PCR)

PCR assays are routinely used for the identification of clonal B-cell populations. These tests amplify the DNA between primers that target the conserved framework (FR) and joining (J) regions (IGH Tubes A-C), the variable (V) and joining (J) regions (IGK Tube A) and the variable, Jκ-Cκ intron, and Kde regions (IGK Tube B). 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 chain genes 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. This Gaussian distribution reflects the heterogeneous population of V-J rearrangements. 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. Depicted is a simple representation of the organization of a rearranged immunoglobulin heavy chain (IGH) gene on chromosome 14 and the immunoglobulin kappa light chain gene on chromosome 2p11.2. Black arrows represent the relative positions of primers that target the conserved framework (FR1-3) and the downstream consensus JH gene segments for IGH and the Vk, Jκ, INTR and Kde primers which are included in the IGK master mix tubes. The amplicon products generated from each of these regions can be differentially detected when fluorescent primer sets are used with capillary electrophoresis instruments that employ differential fluorescence detection.

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. Note that the primers that amplify the different FR regions, which are located at three distinct sections along the heavy chain gene, produce a correspondingly different size-range of V-J products. For rearrangements of the IGK gene, the
length of the CDR3 region is limited and display significant skewing (platykurtosis). Thus, PCR products display a very narrow Gaussian distribution and are most easily and reliably identified by heteroduplex analysis.

4.2. 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 (fluorophors) 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 nucleotides. 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

<table>
<thead>
<tr>
<th>Catalog #</th>
<th>Catalog Name</th>
<th>Reagent Components (active ingredients)</th>
<th>Unit</th>
<th>9-100-0031 # of Units</th>
<th>9-100-0041 # of Units</th>
<th>Storage Temp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-101-0011</td>
<td>IGH Tube A – 6FAM</td>
<td>Multiple oligonucleotides targeting the framework 1 region of the immunoglobulin heavy chain gene in a buffered salt solution.</td>
<td>1500 µL</td>
<td>1</td>
<td>10</td>
<td>-65 to -85°C</td>
</tr>
<tr>
<td>2-101-0101</td>
<td>IGH Tube B – 6FAM</td>
<td>Multiple oligonucleotides targeting the framework 2 region of the immunoglobulin heavy chain gene in a buffered salt solution.</td>
<td>1500 µL</td>
<td>1</td>
<td>10</td>
<td>-65 to -85°C</td>
</tr>
<tr>
<td>2-101-0031</td>
<td>IGH Tube C – HEX</td>
<td>Multiple oligonucleotides targeting the framework 3 region of the immunoglobulin heavy chain gene in a buffered salt solution.</td>
<td>1500 µL</td>
<td>1</td>
<td>10</td>
<td>-65 to -85°C</td>
</tr>
<tr>
<td>2-102-0011</td>
<td>IGK Tube A – 6FAM</td>
<td>Multiple oligonucleotides targeting the variable and joining regions of the immunoglobulin kappa light chain gene in a buffered salt solution.</td>
<td>1500 µL</td>
<td>1</td>
<td>10</td>
<td>-65 to -85°C</td>
</tr>
<tr>
<td>2-102-0021</td>
<td>IGK Tube B – 6FAM</td>
<td>Multiple oligonucleotides targeting the variable, Jκ-Cκ intron, and Kde regions of the immunoglobulin kappa light chain gene in a buffered salt solution.</td>
<td>1500 µL</td>
<td>1</td>
<td>10</td>
<td>-65 to -85°C</td>
</tr>
<tr>
<td>2-096-0021</td>
<td>Specimen Control Size Ladder – 6FAM</td>
<td>Multiple oligonucleotides targeting housekeeping genes.</td>
<td>1500 µL</td>
<td>1</td>
<td>10</td>
<td>-65 to -85°C</td>
</tr>
<tr>
<td>4-088-1750</td>
<td>IVS-0030 Clonal Control DNA</td>
<td>200 µg/mL of DNA in 1/10th TE solution</td>
<td>100 µL</td>
<td>1</td>
<td>5</td>
<td>2 to 8°C or -65 to -85°C</td>
</tr>
<tr>
<td>4-088-1090</td>
<td>IVS-0019 Clonal Control DNA</td>
<td>200 µg/mL of DNA in 1/10th TE solution</td>
<td>100 µL</td>
<td>1</td>
<td>5</td>
<td>2 to 8°C or -65 to -85°C</td>
</tr>
<tr>
<td>4-088-0370</td>
<td>IVS-0007 Clonal Control DNA</td>
<td>200 µg/mL of DNA in 1/10th TE solution</td>
<td>100 µL</td>
<td>1</td>
<td>5</td>
<td>2 to 8°C or -65 to -85°C</td>
</tr>
<tr>
<td>4-092-0010</td>
<td>IVS-0000 Polyclonal Control DNA</td>
<td>200 µg/mL of DNA in 1/10th TE solution</td>
<td>100 µL</td>
<td>1</td>
<td>5</td>
<td>2 to 8°C or -65 to -85°C</td>
</tr>
</tbody>
</table>

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, 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.
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 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 DNA controls is 2°C to 8°C, but DNA controls can 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 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.

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 310, ABI 3100, or ABI 3130**

- 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 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)
- Follow manufacturer’s installation, operation, calibration, and maintenance procedures.
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:
1. Divalent cation chelators
2. Low retention pipette tips
3. EDTA (not significant at low concentrations)
4. Heparin

7.3. Specimen Requirements and Handling
This assay tests genomic DNA from the following sources:
1. 5cc 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)
2. Minimum 5mm cube of tissue (stored and shipped frozen; or stored and shipped in RPMI 1640 at ambient temperature or on ice)
3. 2μg of genomic DNA (stored at 2°C to 8°C and shipped at ambient temperature)
4. Formalin-fixed paraffin embedded tissue or slides (stored and shipped at ambient temperature)

7.4. 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.

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>
<thead>
<tr>
<th>Catalog #</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-101-0011</td>
<td>IGH Tube A – 6FAM</td>
</tr>
<tr>
<td>2-101-0101</td>
<td>IGH Tube B – 6FAM</td>
</tr>
<tr>
<td>2-101-0031</td>
<td>IGH Tube C – HEX</td>
</tr>
<tr>
<td>2-102-0011</td>
<td>IGK Tube A – 6FAM</td>
</tr>
<tr>
<td>2-102-0021</td>
<td>IGK Tube B – 6FAM</td>
</tr>
<tr>
<td>2-096-0021</td>
<td>Specimen Control Size Ladder – 6FAM</td>
</tr>
<tr>
<td>4-088-1750</td>
<td>IVS-0030 Clonal Control DNA</td>
</tr>
<tr>
<td>4-088-1090</td>
<td>IVS-0019 Clonal Control DNA</td>
</tr>
<tr>
<td>4-088-0370</td>
<td>IVS-0007 Clonal Control DNA</td>
</tr>
<tr>
<td>4-092-0010</td>
<td>IVS-0000 Polyclonal Control DNA</td>
</tr>
</tbody>
</table>
8.2. Materials Required But Not Provided

<table>
<thead>
<tr>
<th>Reagent/Material</th>
<th>Recommended Reagents/Materials and Suppliers</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA Polymerase</td>
<td>Applied Biosystems: <em>AmpliTaq Gold®</em> DNA Polymerase (Cat# N808-0241)</td>
<td>N/A</td>
</tr>
<tr>
<td>Glass Distilled De-ionized Molecular Biology Grade or USP Water</td>
<td>N/A</td>
<td>Water should be sterile and free of DNase and RNase.</td>
</tr>
<tr>
<td>Calibrated Pipettes</td>
<td>Rainin: P-2, P-20, P-200, and P-1000 pipettes Or SL-2, SL-20, SL-200, and SL-1000 pipettes</td>
<td>Must be able to accurately measure volumes between 1μl and 1000μl.</td>
</tr>
<tr>
<td>Thermocycler</td>
<td>Bio-Rad: PTC-100 or PTC-200, PTC-220, PTC-240 Perkin-Elmer PE 9600 or PE 9700</td>
<td>N/A</td>
</tr>
<tr>
<td>Vortex Mixer</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>PCR plates or tubes</td>
<td>N/A</td>
<td>Sterile</td>
</tr>
<tr>
<td>Filter barrier pipette tips</td>
<td>N/A</td>
<td>Sterile, RNase/DNase/Pyrrogen-free</td>
</tr>
<tr>
<td>Microcentrifuge tubes</td>
<td>N/A</td>
<td>Sterile</td>
</tr>
<tr>
<td>ABI Capillary Electrophoresis Instrument</td>
<td>Applied Biosystems: ABI 310, 3100, or 3130 series</td>
<td>N/A</td>
</tr>
<tr>
<td>Hi-Di Formamide</td>
<td><em>InVivoScribe Technologies</em>: HI-Deionized Formamide (Cat# 6-098-0041)</td>
<td>N/A</td>
</tr>
<tr>
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<td>Applied Biosystems: Hi-Di™ Formamide (Cat# 4311320)</td>
<td></td>
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<tr>
<td>ROX Size Standards</td>
<td><em>InVivoScribe Technologies</em>: Hi-Di Formamide w/ROX size standards for ABI 310 (Cat# 6-098-0051)</td>
<td>N/A</td>
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<td></td>
<td>Hi-Di Formamide w/ROX size standards for ABI 3100 (Cat# 6-098-0061)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Applied Biosystems: *GeneScan™ - 400HD [ROX]™ (Cat# 402985)</td>
<td></td>
</tr>
<tr>
<td>Spectral Calibration Dye Set D</td>
<td>Applied Biosystems: For ABI 3100 and 3130 instruments: DS-30 Matrix Standard Kit (Dye Set D) (Cat# 4345827)</td>
<td>Dye set used to spectrally calibrate ABI instrument for use with 6FAM, HEX, NED, and ROX</td>
</tr>
<tr>
<td></td>
<td>For ABI 310 instruments: NED Matrix Standard (Cat# 402996) And Fluorescent Amidite Matrix Standards [6FAM, TET, HEX, TAMRA, ROX] (Cat# 401546)</td>
<td></td>
</tr>
<tr>
<td>Polymer</td>
<td>Applied Biosystems: POP-4 Polymer: POP-4™ for 310 Genetic Analyzers (Cat# 402838)</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>POP-4™ for 3100/3100-Avant Genetic Analyzers (Cat# 4316355)</td>
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<tr>
<td></td>
<td>POP-4™ for 3130/3130XL Genetic Analyzers (Cat# 4352755)</td>
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<tr>
<td></td>
<td>POP-7 Polymer: POP-7™ for 3130/3130XL Genetic Analyzers (Cat# 4352759)</td>
<td></td>
</tr>
<tr>
<td>Buffer</td>
<td>Applied Biosystems: 10X Genetic Analyzer Buffer with EDTA (Cat# 402824)</td>
<td>Dilute 1:10 in sterile water before use</td>
</tr>
</tbody>
</table>

8.3. Reagent Preparation

- All unknown 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.
- 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.
- Positive, negative and no template controls should be tested for each of the master mixes.

1. Using gloved hands, remove the master mixes from the freezer. Allow the tubes to thaw completely; then gently vortex to mix.
2. In containment hood or dead air box remove an appropriate aliquot from each master mix to individual clean, sterile microcentrifuge tubes. Aliquot volumes should be 45μl for each reaction. We recommend adding 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 \( (n) \) should be:

\[
\begin{align*}
n &= 2 \times \text{# of samples} \quad \text{(run each sample in duplicate)} \\
&+ 1 \quad \text{positive control DNA (See section 8.7 Recommended Positive Controls)} \\
&+ 1 \quad \text{negative control DNA (IVS-0000 Polyclonal Control DNA)} \\
&+ 1 \quad \text{no template control (water)} \\
&+ 1 \quad \text{to correct for pipetting errors}
\end{align*}
\]

\[
n = 2 \times \text{# of samples} + 4 \quad \text{Total}
\]

Therefore the total aliquot volume for each master mix should be \( n \times 45\mu l \).

For the Specimen Control Size Ladder master mix, the number of reactions \( (m) \) should be:

\[
\begin{align*}
m &= \text{# of samples} \quad \text{(run each sample in singlicate)} \\
&+ 1 \quad \text{positive control DNA (IVS-0000 Polyclonal Control DNA)} \\
&+ 1 \quad \text{no template control (water)} \\
&+ 1 \quad \text{to correct for pipetting errors}
\end{align*}
\]

\[
m = \text{# of samples} + 3 \quad \text{Total}
\]

Therefore the total aliquot volume for the Specimen Control Size Ladder master mix should be \( m \times 45\mu l \).

3. Add 1.25 units (or 0.25\mu l at 5units/\mu l) of AmpliTaq Gold DNA polymerase per reaction to each master mix. The total AmpliTaq Gold DNA polymerase added to each master mix should be \( n \times 0.25\mu l \), and \( m \times 0.25\mu l \) for the Specimen Control Size Ladder master mix. Gently vortex to mix.

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

5. Add 5\mu l 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.

- 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:

- \( n \times 45\mu l \) Master Mix
- \( n \times 0.25\mu l \) AmpliTaq Gold DNA polymerase

Vortex gently to mix.

Aliquot \( 45\mu l \) of master mix + DNA polymerase solution into each reaction well.

Add \( 5\mu l \) of appropriate Template to each well.

Total reaction volume = \( 50\mu l \)

### 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 PTC thermocyclers.)*

   **Standard Program for AmpliTaq Gold**

   - 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

   IdentiClone™ IGH + IGK B-Cell Clonality Assay – ABI Fluorescence Detection
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 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-nucleotide peak has a preceding peak that shows up at 84 nucleotides.

- We do not recommend multiplexing of PCR products from different master mixes as this will result in overall reduced sensitivity of the assay.

1. In a new microcentrifuge tube, mix an appropriate amount (for a total of 10μl per PCR reaction) of Hi-Di Formamide with ROX Size Standards\. Vortex well.
2. In a new 96-well PCR plate, add 10μl of Hi-Di Formamide with ROX size standards to individual wells for each PCR reaction.
3. Transfer 1μl 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 or tubes.
5. Heat denature the samples at 95°C 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 capillary electrophoresis instrument according to the 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.)

Note\a: Please see Applied Biosystems’ accompanying product insert for mixing Hi-Di Formamide with ROX size standards for different ABI instruments. Alternatively, pre-mixed aliquots may be purchased directly from InvivoScribe Technologies.

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 1-, 4-, and 16-capillary instruments this is equal to 48, 192, and 768 samples per day, respectively), and automatically analyze and store data for review or comparison with other test results.

8.6. Quality Control

Positive and negative (or normal) controls are furnished with the kit and should be run in singlicate each time the assay is performed to ensure proper performance of the assay. In addition a no template control (e.g. water) should also be included to test for contamination of the master mix or cross-contamination of PCR 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 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.

8.7. Recommended Positive Controls

- The amplicon sizes listed were determined using an ABI 3100 platform. Amplicon sizes seen on your specific capillary electrophoresis instrument may differ 1 to 4 nucleotides (nt) 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. The amplicon sizes listed were determined using an ABI 3100 platform.
Table 4

<table>
<thead>
<tr>
<th>Master Mix</th>
<th>Target</th>
<th>Color</th>
<th>Control DNA</th>
<th>Catalog #</th>
<th>Product Size in Nucleotides²</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGH Tube A</td>
<td>FR1-JH</td>
<td>Blue</td>
<td>Valid Size Range</td>
<td>4-088-1750</td>
<td>310-360, 280, 325</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>IVS-0030 Clonal Control DNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGH Tube B</td>
<td>FR2-JH</td>
<td>Blue</td>
<td>Valid Size Range</td>
<td>4-088-1750</td>
<td>250-295</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>IVS-0030 Clonal Control DNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGH Tube C</td>
<td>FR3-JH</td>
<td>Green</td>
<td>Valid Size Range</td>
<td>4-088-1090</td>
<td>100-170</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>IVS-0019 Clonal Control DNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGK Tube A</td>
<td>Vκ-Jκ</td>
<td>Blue</td>
<td>Valid Size Range</td>
<td>4-088-0370</td>
<td>120-160, 190-210, 260-300</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>IVS-0007 Clonal Control DNA</td>
<td></td>
<td>143</td>
</tr>
<tr>
<td>IGK Tube B</td>
<td>Vκ-Kde + intron-Kde</td>
<td>Blue</td>
<td>Valid Size Range</td>
<td>4-088-0370</td>
<td>210-250, 270-300, 350-390</td>
</tr>
<tr>
<td>Specimen Control</td>
<td>Size Ladder</td>
<td>Multiple Genes</td>
<td>Blue</td>
<td>Valid Size Range</td>
<td>4-092-0010</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>IVS-0000 Polyclonal Control DNA</td>
<td></td>
<td>100, 200, 300, 400, 600⁷</td>
</tr>
</tbody>
</table>

Note 1: A 280bp band may also be present and is a known amplicon that lies just outside the valid size range for IGH Tube A.
Note 2: Because smaller PCR fragments are preferentially amplified, it is not unusual for the 600bp fragment to have a diminished signal or to be missing entirely.

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 each of the master mixes 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 of the master mixes.

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 there was DNA of insufficient quantity or quality for analysis”.
2. Samples that test negative should be repeated if the positive control reaction failed.
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 should not be interpreted.

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

Table 5

<table>
<thead>
<tr>
<th>Type of Control</th>
<th>Expected Result</th>
<th>Aberrant Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Template Control</td>
<td>No amplification present, continue with analysis</td>
<td>Amplification present, Repeat the assay.</td>
</tr>
<tr>
<td>Polyclonal Control</td>
<td>Product size is consistent with expected size listed in section 11.1 Expected Size of Amplified Products. No clonal rearrangements are present. Continue with analysis.</td>
<td>Clonal rearrangements are present. Repeat the assay.</td>
</tr>
<tr>
<td>Positive Control (This can also</td>
<td>Product size is consistent with expected size listed in section 11.1 Expected Size of Amplified Products. Continue with analysis.</td>
<td>Repeat the assay.</td>
</tr>
<tr>
<td>be an extraction control if positive control material is taken through extraction processes)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Specimen Control Size Ladder (This amplification control is essential for samples of unknown quantity and quality.)</td>
<td>If all of the 100, 200, 300, 400, and 600bp peaks are seen, continue with analysis. Because smaller PCR fragments are preferentially amplified, it is not unusual for the 600bp fragment to have a diminished signal or to be missing entirely. Continue with analysis.</td>
<td>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.</td>
</tr>
</tbody>
</table>
9.2. **Sample Interpretation**  
Given that the controls produce expected results, the clinical samples should be interpreted as follows:

- One or two prominent positive bands within the valid size range are reported as:

  “Positive for the detection of clonal immunoglobulin heavy chain or kappa light 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.”

- An absence of positive bands within the valid size range is reported as:

  “Negative for the detection of clonal immunoglobulin heavy chain or kappa light chain gene rearrangement(s).”

**Note a:** Criteria for defining a positive band 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.

10. **Limitations of Procedure**

- This assay does not identify 100% of clonal cell populations.
- This assay cannot reliably detect less than 1 positive cells per 100 normal cells.
- The results of molecular clonality tests should always be interpreted 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.
11. Expected Values

11.1. Expected Size of Amplified Products

- The amplicon sizes listed were determined using an ABI 3100 platform. Amplicon sizes seen on your specific capillary electrophoresis instrument may differ 1 to 4 nucleotides (nt) 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. The amplicon sizes listed were determined using an ABI 3100 platform.

<table>
<thead>
<tr>
<th>Master Mix</th>
<th>Target</th>
<th>Color</th>
<th>Control DNA</th>
<th>Catalog #</th>
<th>Product Size in Nucleotides</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGH Tube A</td>
<td>FR1-JH</td>
<td>Blue</td>
<td>Valid Size Range</td>
<td>4-092-0010</td>
<td>310-360</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>IVS-0000 Polyclonal Control DNA</td>
<td>4-088-1750</td>
<td>310-360</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>IVS-0030 Clonal Control DNA</td>
<td>4-088-1090</td>
<td>280(^a), 325</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>IVS-0019 Clonal Control DNA</td>
<td></td>
<td>345</td>
</tr>
<tr>
<td>IGH Tube B</td>
<td>FR2-JH</td>
<td>Blue</td>
<td>Valid Size Range</td>
<td>4-092-0010</td>
<td>250-295</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>IVS-0000 Polyclonal Control DNA</td>
<td>4-088-1750</td>
<td>250-295</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>IVS-0030 Clonal Control DNA</td>
<td>4-088-1090</td>
<td>260</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>IVS-0019 Clonal Control DNA</td>
<td></td>
<td>285</td>
</tr>
<tr>
<td>IGH Tube C</td>
<td>FR3-JH</td>
<td>Green</td>
<td>Valid Size Range</td>
<td>4-092-0010</td>
<td>100-170</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>IVS-0000 Polyclonal Control DNA</td>
<td>4-088-1750</td>
<td>100-170</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>IVS-0030 Clonal Control DNA</td>
<td>4-088-1090</td>
<td>---</td>
</tr>
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<td></td>
<td></td>
<td>IVS-0019 Clonal Control DNA</td>
<td></td>
<td>145</td>
</tr>
<tr>
<td>IGK Tube A</td>
<td>V kappa-J kappa</td>
<td>Blue</td>
<td>Valid Size Range</td>
<td>4-092-0010</td>
<td>120-160, 190-210, 260-300</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>IVS-0000 Polyclonal Control DNA</td>
<td>4-088-0370</td>
<td>135-155(^a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>IVS-0030 Clonal Control DNA</td>
<td></td>
<td>143</td>
</tr>
<tr>
<td>IGK Tube B</td>
<td>V kappa-Kde + intron-Kde</td>
<td>Blue</td>
<td>Valid Size Range</td>
<td>4-092-0010</td>
<td>210-250, 270-300, 350-390</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>IVS-0000 Polyclonal Control DNA</td>
<td>4-088-0370</td>
<td>225-245, 265-285, 404(^b,c)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>IVS-0007 Clonal Control DNA</td>
<td></td>
<td>274, 282</td>
</tr>
<tr>
<td>Specimen Control Size Ladder</td>
<td>Multiple Genes</td>
<td>Blue</td>
<td>Valid Size Range</td>
<td>4-092-0010</td>
<td>100, 200, 300, 400, 600(^d)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>IVS-0000 Polyclonal Control DNA</td>
<td></td>
<td>100, 200, 300, 400, 600(^d)</td>
</tr>
</tbody>
</table>

**Note**: A 280bp band may also be present and is a known amplicon that lies just outside the valid size range for IGH Tube A.

**Note**: The normal distribution for IGK gene rearrangements is highly truncated due to limited junctional diversity. Please refer to the paper by Rock et al. for a detailed explanation.

**Note**: Under sub-optimal conditions an aspecific product of 404bp can be detected in Tube B. To discriminate between specific and aspecific, negative control DNA should not show this band within the same experiment. If a band is present, we then consider the band aspecific.

**Note**: Because smaller PCR fragments are preferentially amplified, it is not unusual for the 600bp fragment to have a diminished signal or to be missing entirely.
11.2. **Sample Data**
The data shown below were generated using the master mixes indicated. Amplified products were run on an ABI 3100 instrument.

For each master mix:
- Panel 1 displays data generated testing an alternative 100% clonal control DNA.
- Panel 2 displays data generated testing the recommended 100% clonal control DNA.
- Panel 3 displays data generated testing a 10% dilution of the recommended clonal control DNA.
- Panel 4 displays data generated testing IVS-0000 Polyclonal Control DNA.

*Figure 2*

*Figure 3*
Figure 4

Figure 5
Figure 6

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 recommended positive control, IVS-0000 Polyclonal Control DNA.
- Panels 3 and 4 display data generated testing two different 100% clonal control DNAs.

Figure 7

Table 7

<table>
<thead>
<tr>
<th>PCR/SB concordance (Leukemia²):</th>
<th>PCR/SB concordance (JMD³):</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGH: 93% sensitivity/92% specificity</td>
<td>IGH + IGK: 85% sensitivity</td>
</tr>
<tr>
<td>IGK: 90% sensitivity/90% specificity</td>
<td></td>
</tr>
<tr>
<td>IGL: 86% sensitivity/92% specificity</td>
<td></td>
</tr>
<tr>
<td>TCRB: 86% sensitivity/98% specificity</td>
<td>TCRB: 85% sensitivity</td>
</tr>
<tr>
<td>TCRG: 89% sensitivity/94% specificity</td>
<td></td>
</tr>
</tbody>
</table>

12. Performance Characteristics

This IdentiClone™ IGH + IGK B-Cell 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.
TCRD: 83% sensitivity/ 95% specificity

PCR vs. SB analysis relative to histopathology and final diagnosis:

Table 8

<table>
<thead>
<tr>
<th></th>
<th>PCR/ SB concordance</th>
<th>PCR sensitivity</th>
<th>SB sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGH + IGK:</td>
<td>85%</td>
<td>98%</td>
<td>39%</td>
</tr>
<tr>
<td>TCRB:</td>
<td>85%</td>
<td>96%</td>
<td>35%</td>
</tr>
</tbody>
</table>

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 precision4. 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.

13. Bibliography


14. Technical and Customer Service

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

15. Legal Notice

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These methods also require nucleic acid amplification methods such as Polymerase Chain Reaction (PCR). In certain countries, this technology is or may be covered by patents owned by Hoffmann-LaRoche, Inc. and F. Hoffmann-LaRoche Ltd. No license under any such patents to use the PCR process is conveyed expressly or by implication to the purchaser by the purchase of these products.

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