IdentiClone®

Instructions for Use

IdentiClone® *IGH* + *IGK* B-Cell Clonality Assay

CE UK CA IVD

For identification of clonal immunoglobulin heavy chain and kappa light 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#	Products	Quantity
REF 91000031	IdentiClone *IGH* + *IGK* B-Cell Clonality Assay – ABI Fluorescence Detection	33 Reactions
REF 91000041	IdentiClone *IGH* + *IGK* B-Cell Clonality Assay MegaKit – ABI Fluorescence Detection	330 Reactions

Table of Contents

1. 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[4]
- 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

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 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.

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 testing of more than 400 clinical samples using Revised European/American Lymphoma (REAL) classification. Testing was performed 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 indicate that *IGH* and *IGK* gene rearrangements may improve the reliability and sensitivity of the tests.[2] In addition, testing for both *IGH* and *IGK* gene rearrangements led to 99% sensitivity, compared to 88% for *IGH* and 88% for *IGK* when tested alone, which 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.[4]

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.

2.2. Summary

This test kit includes six (6) master mixes. The *IGH* Tubes A, B, and C master mixes target the framework 1, 2, and 3 regions (respectively) within the variable and joining regions 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 (K_{de}) rearrangements with the variable (V) region and the intragenic Jκ-Cκ region. The resulting Vκ-K_{de} and Jκ-Cκ intron-K_{de} 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 96, 197, 297, 397 and 602 nucleotides (nt) 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 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 K_{de} 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 generated, reflecting the heterogeneous population of V-J rearrangements. DNA from samples containing a clonal population yield one or two prominent amplified products (amplicons) within a diminished polyclonal background.

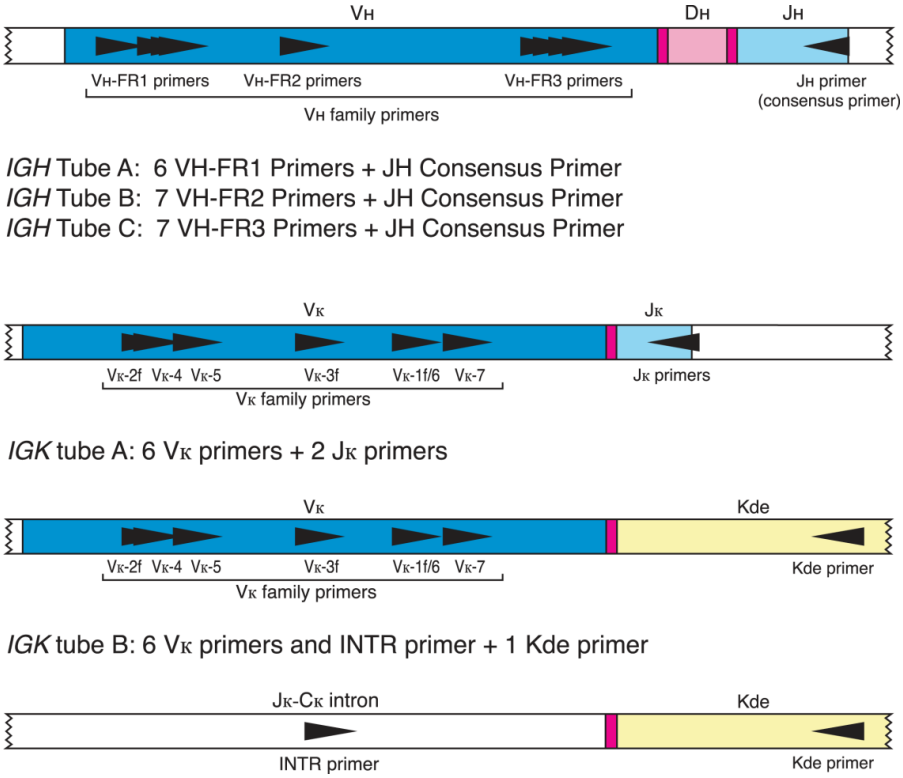

Figure 1. Depicted is a simple representation of the organization of a rearranged immunoglobulin heavy chain *(IGH)* gene on chromosome 14 and the immunoglobulin kappa light chain gene on chromosome 2p11.2. Black arrows represent the relative positions of primers that target the conserved framework (FR1-3) and the downstream consensus J_H gene segments for *IGH* and the Vκ, Jκ, INTR and K_{de} 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 various-sized amplicon products that form a Gaussian distribution around a statistically favored rearrangement. 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.

3.2. Differential Flourescence 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.

4. Reagents

4.1. Reagent Components

Table 1: Available Assays

Catalog Number	Description	Quantity
REF 91000031	IdentiClone *IGH* + *IGK* B-Cell Clonality Assay – ABI Fluorescence Detection	33 Reactions
REF 91000041	IdentiClone *IGH* + *IGK* B-Cell Clonality Assay MegaKit – ABI Fluorescence Detection	330 Reactions

Table 2. Kit Components

Reagent	Catalog #	Reagent Components (active ingredients)	Unit Quantity	91000031 # of Units	91000041 # of Units	Storage Temp.
Master Mixes	21010011CE	***IGH* Tube A – 6FAM** Multiple oligonucleotides targeting the framework 1 region of the immunoglobulin heavy chain gene in a buffered salt solution.	1500 µL	1	10	-85°C to -65°C
	21010101CE	***IGH* Tube B – 6FAM** Multiple oligonucleotides targeting the framework 2 region of the immunoglobulin heavy chain gene in a buffered salt solution.	1500 µL	1	10	
	21010031CE	***IGH* Tube C – HEX** Multiple oligonucleotides targeting the framework 3 region of the immunoglobulin heavy chain gene in a buffered salt solution.	1500 µL	1	10	
	21020011CE	***IGK* Tube A - 6FAM** Multiple oligonucleotides targeting the variable and joining regions of the immunoglobulin kappa light chain gene in a buffered salt solution.	1500 µL	1	10	
	21020021CE	***IGK* Tube B - 6FAM** Multiple oligonucleotides targeting the variable, Jκ-Cκ intron, and K_{de} regions of the immunoglobulin kappa light chain gene in a buffered salt solution.	1500 µL	1	10	
Template Amplification Control Master Mix	20960021	**Specimen Control Size Ladder – 6FAM** Multiple oligonucleotides targeting housekeeping genes.	1500 µL	1	10	
Positive Control DNAs	40881750	**IVS-0030 Clonal Control DNA** 200 µg/mL of DNA in 1/10th TE solution	100 µL	1	5	2°C to 8°C or -85°C to -65°C
	40881090	**IVS-0019 Clonal Control DNA** 200 µg/mL of DNA in 1/10th TE solution	100 µL	1	5	
	40880370	**IVS-0007 Clonal Control DNA** 200 µg/mL of DNA in 1/10th TE solution	100 µL	1	5	
Negative (Normal) Control DNA	40920010	**IVS-0000 Polyclonal Control DNA** 200 µg/mL of DNA in 1/10th 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 this 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. Use care to ensure use of correct thermal cycler program, 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.
- 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 workflow in the PCR laboratory; begin with master mix preparation, move to specimen preparation, then to amplification, and finally to detection. Do not bring amplified DNA into the areas designated for master mix or specimen preparation.
- Dedicate 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, and for long term storage 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
- 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)
    - 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)
- 3 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. Materials Provided

Table 3. Kit Components

Catalog #	Description
21010011CE	*IGH* Tube A – 6FAM
21010101CE	*IGH* Tube B – 6FAM
21010031CE	*IGH* Tube C – HEX
21020011CE	*IGK* Tube A – 6FAM
21020021CE	*IGK* Tube B – 6FAM
20960021	Specimen Control Size Ladder – 6FAM
40881750	IVS-0030 Clonal Control DNA
40881090	IVS-0019 Clonal Control DNA
40880370	IVS-0007 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: • EagleTaq DNA Polymerase Invivoscribe, Inc.: • FalconTaq DNA Polymerase or equivalent	05206944190 60970130	N/A
Glass Distilled De-ionized Molecular Biology Grade or USP Water	N/A	N/A	Sterile and free of DNase and RNase.
Calibrated Pipettes	Rainin: • P-2, P-20, P-200, and P-1000 pipettes • 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: • Veriti Dx Thermal Cycler Bio-Rad: • PTC-100 or PTC-200, PTC-220, PTC-240 Perkin-Elmer • 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	Applied Biosystems: • ABI 310, 3100, or 3500 series	N/A	N/A
Hi-Di Formamide	Applied Biosystems: • Hi-Di™ Formamide	4311320	N/A

Size Standards	Invivoscribe:
	• Hi-Di Formamide w/ROX size standards for ABI 3100
	Applied Biosystems:
	• For ABI 3100 or 3130 instruments:
	  ○ GeneScan™ - 400HD [ROX]™
	• For ABI 3500 instruments:
	  ○ GeneScan - 600 [LIZ]™ v2.0
		60980061
		
		402985
		
		4408399
			N/A

Spectral Calibration Dye Sets	Applied Biosystems:
	• For ABI 3100 and 3130 instruments:
	  ○ DS-30 Matrix Standard Kit (Dye Set D)
	• For ABI 310 instruments:
	  ○ NED Matrix Standard and Fluorescent Amidite Matrix Standards [6FAM, TET, HEX, TAMRA, ROX]
		4345827
		
		401546
		402996
			N/A

Table 4. Materials Required (not provided)

Reagent/Material	Recommended Reagents/Materials and Suppliers	Catalog #	Notes
Spectral Calibration Dye Sets	• For ABI 3500 instruments: ○ DS-33 Matrix Standard Kit (Dye Set G5)	4345833	N/A

Polymer	Applied Biosystems:
	• POP-4™ Polymer:
	  ○ POP-4 for 310 Genetic Analyzers
	  ○ POP-4 for 3100/3100-Avant Genetic Analyzers
	  ○ POP-4 for 3130/3130xL Genetic Analyzers
	• POP-7™ Polymer:
	  ○ POP-7 for 3130/3130xL Genetic Analyzers
	  ○ POP-7 for 3500/3500xL Genetic Analyzers
		
		402838
		4316355
		4352755
		
		4352759
		4393714
			N/A

Buffer	Applied Biosystems: • 10X Genetic Analyzer Buffer with EDTA	402824	Dilute 1:10 in sterile water before use

7.3. Reagent Preparation

- All unknown samples can be tested using the Specimen Control Size Ladder master mix 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, **duplicate** testing is reccommended 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 = **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 section *7.7 Recommended Positive Controls*)
+ 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 = **n × 45 µL.**
- For the Specimen Control Size Ladder master mix, the number of reactions (**m**) is:

m = # of samples	(run each sample in duplicate)
+ 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 = **m × 45 µL**.

7.3.3. Add 1.25 U (or 0.25 µL @5 U/µL) of Taq DNA polymerase per reaction to each master mix.

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

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

7.3.5. 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.

7.3.6. Cap or cover the PCR plate.

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

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.

- 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

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.

- Multiplexing of PCR products with different master mixes 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. 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.

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

7.5.8. Review profile and controls, report results. (See sections 8: *Interpretation of Results* and 10: *Expected Values,* below.)

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 PCR) of Hi-Di Formamide with LIZ Size Standards. 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 PCR.

7.5.11. Transfer 0.5 µL of each PCR 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.

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

7.5.16. Review profile and controls, report results. (See sections 8: *Interpretation of Results* and 10: *Expected Values,* below.)

7.6. Quality Control

Positive and negative (or normal) controls are furnished with the kit and are to be run in singlicate each time the assay is performed to ensure proper performance of the assay. In addition, include a no template contrsol (*e.g.* water) to test for contamination of the master mix or cross-contamination of PCRs 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 *10.1 Expected Size of Amplified Products*. Additional controls and sensitivity controls (dilutions of positive controls into a 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 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.

Table 6. Expected Sizes of Recommended Positive Controls

Master Mix	Target	Color	Control DNA	Catalog #	Product Size in nucleotides (nt)
***IGH* Tube A**	FR1-J_H	Blue	**Valid Size Range** IVS-0030 Clonal Control DNA	--- 40881750	**310 - 360** 280[a], 325
***IGH* Tube B**	FR2-J_H	Blue	**Valid Size Range** IVS-0030 Clonal Control DNA	--- 40881750	**250 - 295** 260
***IGH* Tube C**	FR3-J_H	Green	**Valid Size Range** IVS-0019 Clonal Control DNA	--- 40881090	**100 - 170** 145
***IGK* Tube A**	Vκ-Jκ	Blue	**Valid Size Range** IVS-0007 Clonal Control DNA	--- 40880370	**120 - 160, 190 - 210, 260 - 300** 143
***IGK* Tube B**	Vκ-K_{de} + intron-K_{de}	Blue	**Valid Size Range** IVS-0007 Clonal Control DNA	--- 40880370	**210 - 250, 270 - 300, 350 - 390** 274, 282
Specimen Control Size Ladder	Multiple Genes	Blue	**Valid Size Range** IVS-0000 Polyclonal Control DNA	--- 40920010	**96, 197, 297, 397, 602**[b] 96, 197, 297, 397, 602[b]

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

[b]Note: Because smaller PCR fragments are preferentially amplified, it is not unusual for the 602 nt fragment to have a diminished signal or to be missing entirely.

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 of the master mixes.

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 testing on 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. If the controls do not yield the correct results, the assay is not valid and the samples cannot be interpreted.

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 (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.	Repeat the assay.
Specimen Control Size Ladder (This amplification control is essential for samples of unknown quantity and quality.)	If all of the 96, 197, 297, 397 and 602 nt peaks are seen, continue with analysis. Because smaller PCR fragments are preferentially amplified, it is not unusual for the 602 nt 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 bands[a] 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[a] within the valid size range is reported as:

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

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

- Products generated from 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 band.
- 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 (1) 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 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.

Table 8. Expected sies of amplified products

Master Mix	Target	Color	Control DNA	Catalog #	Product Size in nt
***IGH* Tube A**	FR1-J_H	Blue	**Valid Size Range** IVS-0000 Polyclonal Control DNA IVS-0030 Clonal Control DNA IVS-0019 Clonal Control DNA	--- 40920010 40881750 40881090	**310 - 360** 310 - 360 280[a], 325 345
***IGH* Tube B**	FR2-J_H	Blue	**Valid Size Range** IVS-0000 Polyclonal Control DNA IVS-0030 Clonal Control DNA IVS-0019 Clonal Control DNA	--- 40920010 40881750 40881090	**250 - 295** 250 - 295 260 285
***IGH* Tube C**	FR3-J_H	Green	**Valid Size Range** IVS-0000 Polyclonal Control DNA IVS-0030 Clonal Control DNA IVS-0019 Clonal Control DNA	--- 40920010 40881750 40881090	**100 - 170** 100 - 170 --- 145
***IGK* Tube A**	Vκ-Jκ	Blue	**Valid Size Range** IVS-0000 Polyclonal Control DNA IVS-0007 Clonal Control DNA	--- 40920010 40880370	**120 - 160, 190 - 210, 260 - 300** 135 - 155 143
***IGK* Tube B**	Vκ-K_{de} + intron-K_{de}	Blue	**Valid Size Range** IVS-0000 Polyclonal Control DNA IVS-0007 Clonal Control DNA	--- 40920010 40880370	**210 - 250, 270 - 300, 350 - 390** 225 - 245, 265 - 285, 404[b,c] 274, 282
Specimen Control Size Ladder	Multiple Genes	Blue	**Valid Size Range** IVS-0000 Polyclonal Control DNA	--- 40920010	**96, 197, 297, 397, 602[d]** 96, 197, 297, 397, 602[d]

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

[b]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.[5]

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

[d]Note: Because smaller PCR fragments are preferentially amplified, it is not unusual for the 602 nt fragment to have a diminished signal or to be missing entirely.

10.2. Sample Data

The data shown below were generated using the master mixes indicated. Amplified products were run on an ABI instrument.

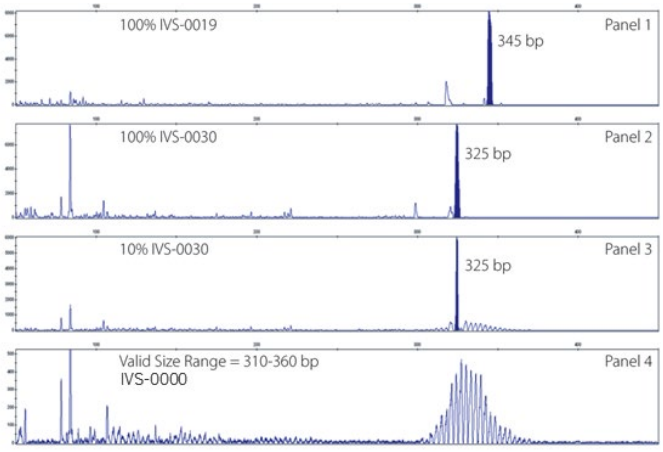

Figure 2. *IGH* Tube A

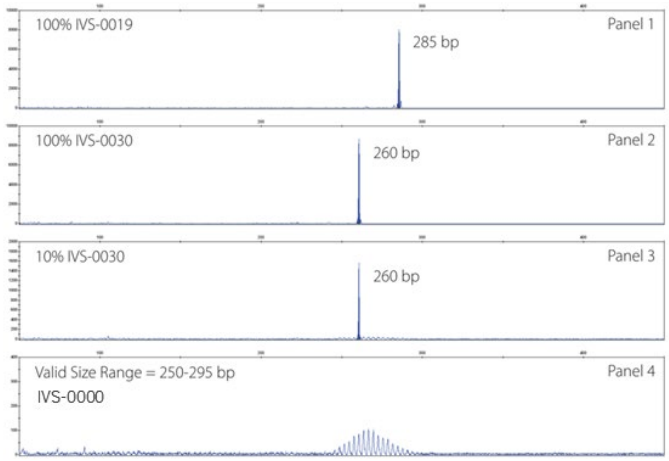

Figure 3. *IGH* Tube B

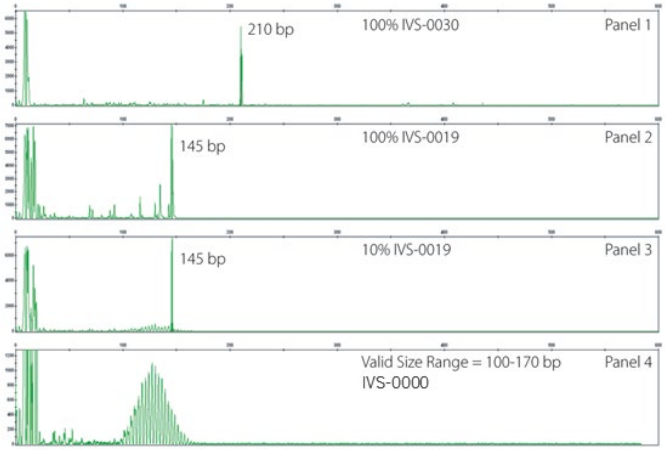

Figure 4. *IGH* Tube C

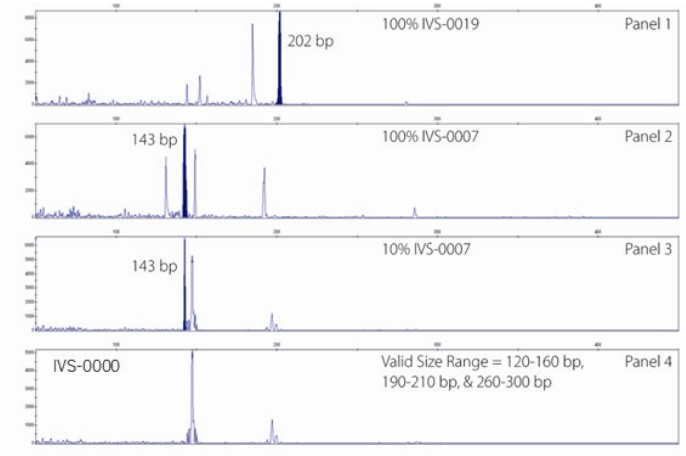

Figure 5. *IGK* Tube A

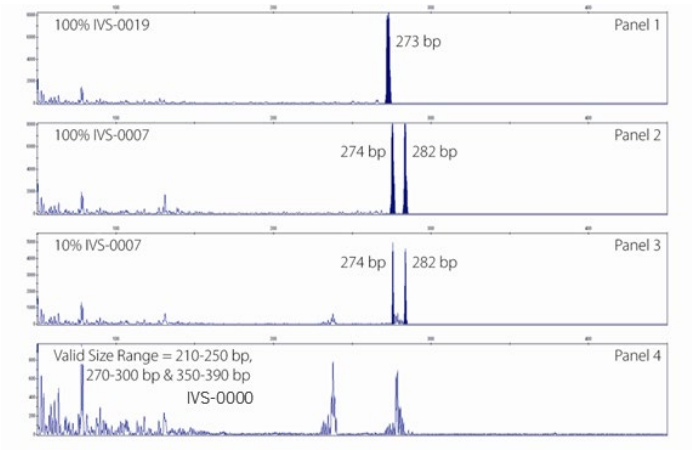

Figure 6. *IGK* Tube B

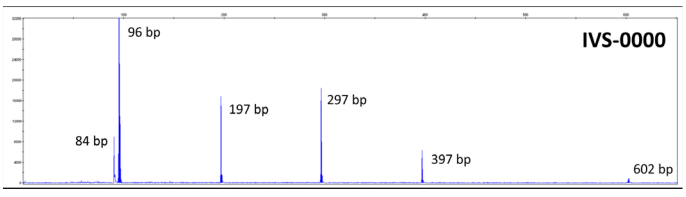

Figure 7. For the Specimen Control Size Ladder master mix

11. 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.[2,3]

Table 9. Concordance Studies

PCR/SB concordance:[2]		PCR/SB concordance:[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%

An independent study by Sandberg *et al.,* included 300 patient samples from a variety of sample types.[3] 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 seven 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. 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.
5. Rock, EP, Sibbald, PR, Davis, MM, Chein, YH. (1994). CDR3 length in antigen-specific immune receptors. *J. Exp. Med*., 179, 323-328.

13. 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

14. Symbols

The following symbols are 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.

Identiclone® is a registered trademark of Invivoscribe®.