

# Instructions for Use




## *IGH* Gene Clonality Assay

For identification of clonal immunoglobulin heavy chain gene rearrangements

**RUO** For RESEARCH USE ONLY. Not for use in diagnostic procedures.

Manufactured in U.S.A.



 Storage Conditions: **-85°C to -65°C**

(DNA controls may be separated from assay kits and stored at 2°C to 8°C)

Catalog #`	Products	Quantity
<b>REF</b> 11010020	<i>IGH</i> Gene Clonality Assay for Gel Detection	33 Reactions
<b>REF</b> 11010061	<i>IGH</i> Gene Clonality Assay for ABI Fluorescence Detection	33 Reactions
<b>REF</b> 11010081	<i>IGH</i> Gene Clonality Assay MegaKit for ABI Fluorescence Detection	330 Reactions

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# 1. Assay Use

This Research Use Only assay identifies immunoglobulin heavy chain clonality and is useful for the study of identifying clonal B-cell populations and evaluation of new research and methods in malignancy studies.

## 2. Summary and Explanation of the Test

### 2.1. Background

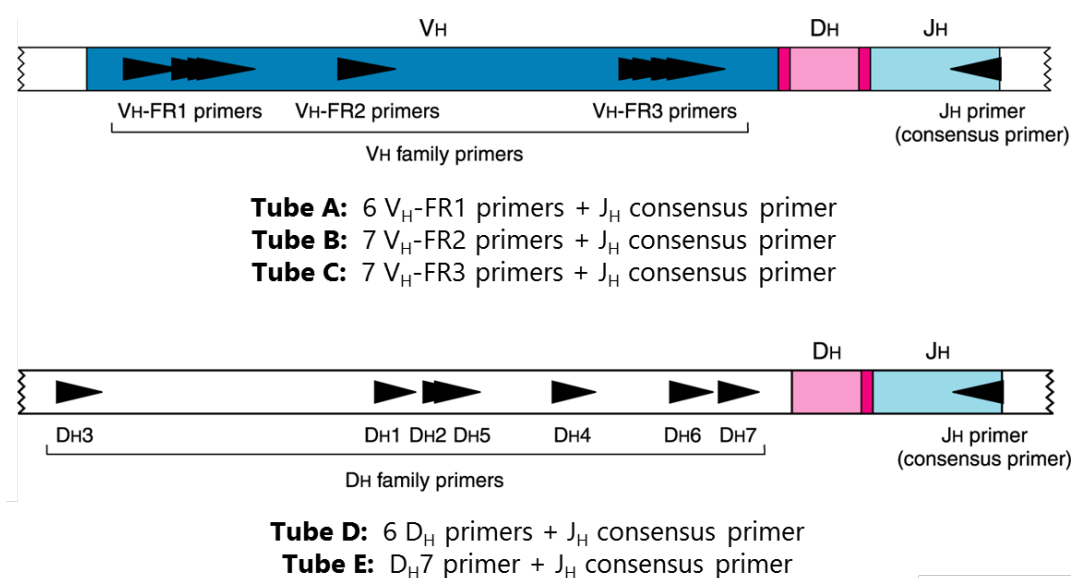
Rearrangements of the antigen receptor genes occur during ontogeny in B and T lymphocytes. These gene rearrangements generate products that are unique in length and sequence for each cell. Therefore, polymerase chain reaction (PCR) assays can be used to identify lymphocyte populations derived from a single cell by detecting the unique V-J gene rearrangements present within these antigen receptor loci.<sup>1</sup> This PCR assay employs multiple consensus DNA primers that target conserved genetic regions within the immunoglobulin heavy chain gene. 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 Gene Rearrangement and Translocation Assays represent a simple approach to PCR-based clonality testing. These standardized assays were carefully optimized testing positive and negative control samples using multiplex master mixes.

### 2.2. Summary

This test kit includes six (6) master mixes. Tubes A, B, and C target the framework 1, 2, and 3 regions (respectively) within the variable ( $V_H$ ) region and the joining region of the Ig heavy chain locus. Tubes D and E target the diversity ( $D_H$ ) and joining ( $J_H$ ) regions. The last master mix, the Specimen Control Size Ladder, targets multiple genes and generates a series of amplicons of 96, 197, 297, 397 and 602 base pairs (bp) to ensure that the quality and quantity of input DNA is adequate to yield a valid result.

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



**Figure 1.** Depicted is a simple representation of the organization of a rearranged immunoglobulin heavy chain gene on chromosome 14. Black arrows represent the relative positions of primers that target the conserved framework (FR1-3) and diversity ( $D_H1$ -7) regions, and the downstream consensus  $J_H$  gene segments. 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 ABI fluorescence detection.

## 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) of the V<sub>H</sub> regions and the conserved J<sub>H</sub> regions (*IGH* Tubes A, B, and C), as well as the D<sub>H</sub> and J<sub>H</sub> regions (*IGH* Tubes D and E). 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<sub>H</sub>-J<sub>H</sub> 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. (In certain cases, where lymphocyte DNA is not present, no product is observed.) For DNA from samples containing a clonal population, the yield is one or two prominent amplified products (amplicons) within a diminished polyclonal background.

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.

### 3.2. Gel Detection

Gel electrophoresis, such as agarose gel electrophoresis or non-denaturing polyacrylamide gel electrophoresis (PAGE), is commonly used to resolve the different amplicon products based on their size, charge, and conformation. Since DNA is negatively charged, when an electrical potential (voltage) is applied across the gel containing PCR products, the electrical field causes the amplicons to migrate through the gel. Smaller DNA fragments are able to easily migrate through the gel matrix, whereas larger DNA fragments migrate more slowly. This causes a separation of the amplicon products based on size. Ethidium bromide or other DNA intercalating dyes can then be used to stain and detect these products in the gel.

A heteroduplex analysis can also be performed and run on a polyacrylamide gel to differentiate clonal and non-clonal PCR products. A heteroduplex analysis involves denaturing the PCR products at a high temperature, then quickly re-annealing the DNA strands by suddenly reducing the temperature. This causes a large portion of DNA strands to incorrectly bind to other non-homologous strands creating loops in the DNA. These loops cause a significant reduction in the ability of the DNA to migrate through a polyacrylamide gel. However, if the majority of the PCR products are clonal, when a heteroduplex analysis is performed, most of these PCR products will correctly re-anneal with a homologous strand. These PCR products will run normally through the polyacrylamide gel. Therefore, in a clonal sample with a polyclonal background, a heteroduplex analysis will cause most of the polyclonal product to run much slower through the polyacrylamide gel, thereby increasing their separation and the ability to identify the clonal band(s).

### 3.3. Differential Fluorescence Detection

Gel electrophoresis is commonly used to resolve the different-sized amplicon products and ethidium bromide or other DNA intercalating dyes to stain and detect these products. A powerful alternative method is use of differential fluorescence detection with primers conjugated with fluorescent dyes that correspond to different targeted regions. Reaction products from several different master mixes can be pooled, fractionated using capillary electrophoresis and detected simultaneously. This detection system results in unsurpassed sensitivity, single base resolution, differential product detection and relative quantification. In addition, the laboratory can eliminate the use of agarose and polyacrylamide gels, as well as the use of carcinogens such as ethidium bromide. Further, differential detection allows accurate, reproducible and objective interpretation of primer-specific products and automatic archiving of data. The automatic archiving of sample data allows comparison of data collected at different times. The inter-assay and intra-assay reproducibility in size determination using capillary electrophoresis is approximately 1-2 base pairs.

## 4. Reagents

### 4.1. Reagent Components

Table 1: Available Kits

Catalog #	Description	Total Reactions
<b>REF</b> 11010020	<i>IGH</i> Gene Clonality Assay – Gel Detection	33 Reactions
<b>REF</b> 11010061	<i>IGH</i> Gene Clonality Assay – ABI Fluorescence Detection	33 Reactions
<b>REF</b> 11010081	<i>IGH</i> Gene Clonality Assay MegaKit – ABI Fluorescence Detection	330 Reactions

Table 2: Kit Components

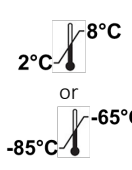
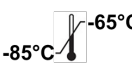
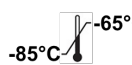

Reagent	Catalog # (REF)	Reagent Components (active ingredients)	Unit Quantity	Assay Kit # of Units	MegaKit # of Units	Storage Temp.
Positive Control DNAs	40881750	<b>IVS-0030 Clonal Control DNA</b> 200 µg/mL of DNA in 1/10th TE solution	100 µL	1	5	
	40881090	<b>IVS-0019 Clonal Control DNA</b> 200 µg/mL of DNA in 1/10th TE solution	100 µL	1	5	
	40881390	<b>IVS-0024 Clonal Control DNA</b> 200 µg/mL of DNA in 1/10th TE solution	100 µL	1	5	
	40880430	<b>IVS-0008 Clonal Control DNA</b> 200 µg/mL of DNA in 1/10th TE solution	100 µL	1	5	
Negative (Normal) Control DNA	40920010	<b>IVS-0000 Polyclonal Control DNA</b> 200 µg/mL of DNA in 1/10th TE solution	100 µL	1	5	
<b>Gel Detection</b>						
Master Mixes for Gel Detection	21010010	<b><i>IGH</i>Tube A – Unlabeled</b> Multiple oligonucleotides targeting the framework 1 region of the immunoglobulin heavy chain gene in a buffered salt solution.	1500 µL	1	N/A	
	21010020	<b><i>IGH</i>Tube B – Unlabeled</b> Multiple oligonucleotides targeting the framework 2 region of the immunoglobulin heavy chain gene in a buffered salt solution.	1500 µL	1	N/A	
	21010030	<b><i>IGH</i>Tube C – Unlabeled</b> Multiple oligonucleotides targeting the framework 3 region of the immunoglobulin heavy chain gene in a buffered salt solution.	1500 µL	1	N/A	
	21010040	<b><i>IGH</i>Tube D – Unlabeled</b> Multiple oligonucleotides targeting the diversity and joining regions of the immunoglobulin heavy chain gene in a buffered salt solution.	1500 µL	1	N/A	
	21010050	<b><i>IGH</i>Tube E – Unlabeled</b> Multiple oligonucleotides targeting the diversity and joining regions of the immunoglobulin heavy chain gene in a buffered salt solution.	1500 µL	1	N/A	
Template Amplification Control Master Mix	20960020	<b>Specimen Control Size Ladder – Unlabeled</b> Multiple oligonucleotides targeting housekeeping genes.	1500 µL	1	N/A	
<b>ABI Fluorescence Detection</b>						
Master Mixes for ABI Fluorescence Detection	21010011	<b><i>IGH</i>Tube A – 6FAM</b> Multiple oligonucleotides targeting the framework 1 region of the immunoglobulin heavy chain gene in a buffered salt solution.	1500 µL	1	10	
	21010101	<b><i>IGH</i>Tube B – 6FAM</b> Multiple oligonucleotides targeting the framework 2 region of the immunoglobulin heavy chain gene in a buffered salt solution.	1500 µL	1	10	

Table 2: Kit Components

Reagent	Catalog # (REF)	Reagent Components (active ingredients)	Unit Quantity	Assay Kit # of Units	MegaKit # of Units	Storage Temp.
Master Mixes for ABI Fluorescence Detection	21010031	<b>IGHTube C – HEX</b> Multiple oligonucleotides targeting the framework 3 region of the immunoglobulin heavy chain gene in a buffered salt solution.	1500 µL	1	10	 -85°C to -65°C
	21010041	<b>IGHTube D – HEX</b> Multiple oligonucleotides targeting the diversity and joining regions of the immunoglobulin heavy chain gene in a buffered salt solution.	1500 µL	1	10	
	21010051	<b>IGHTube E – 6FAM</b> Multiple oligonucleotides targeting the diversity and joining regions of the immunoglobulin heavy chain gene in a buffered salt solution.	1500 µL	1	10	
Template Amplification Control Master Mix	20960021	<b>Specimen Control Size Ladder – 6FAM</b> Multiple oligonucleotides targeting housekeeping genes.	1500 µL	1	10	

## 4.2. Warnings and Precautions

- **RUO** This product is for Research Use Only; not intended for diagnostic procedures.
- 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 thermocycler 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.
- 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, but DNA controls can be stored at -85°C to -65°C long term.
- 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 fluorophores.
- 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. Electrophoresis Unit

- Use or function: DNA fragment separation
- Performance characteristics and specification:
  - Capable of running at 35V to 135V for extended times
- Follow manufacturer's installation, operation, calibration, and maintenance procedures.

### 5.3. UV Illumination Unit

- Use or function: DNA detection
- Performance characteristics and specification:
  - Capable of emitting light at a wavelength of ~302 nm
- Follow manufacturer's installation, operation, calibration, and maintenance procedures.

### 5.4. 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 the specified polymer and capillary type.
- See section 7.5. **Detection** for further details.



## 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 extracted and purified genomic DNA (gDNA). Common sources of gDNA include:

- 5 cc of peripheral blood, bone marrow biopsy or bone marrow aspirate anti-coagulated with heparin or EDTA. Ship at ambient temperature; OR
- Formalin-fixed paraffin embedded tissue or slides

### 6.4. Sample Storage

Store gDNA samples using a method that prevents degradation of DNA.

### 6.5. Sample Preparation

6.5.1. Using any method of DNA extraction, extract the gDNA from unknown samples.

6.5.2. Resuspend DNA to final concentration of 100 µg – 400 µg per mL in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) or distilled 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.
- When testing unknown samples, a template amplification control master mix (*e.g.*, Amplification Control or Specimen Control Size ladder) can be used to verify the absence of PCR inhibitors and sufficient quality and quantity of DNA is present to generate a valid result.

### 6.6. Available Template Amplification Controls

The **Specimen Control Size Ladder** master mix primers are available unlabeled for gel detection and labeled with a fluorescent dye (6-FAM) for ABI fluorescence detection. The label is detected as **BLUE** using the differential fluorescence software. The amplicons produced with this master mix are at 96, 197, 297, 397 and 602 base pair (bp).

- The ~96 bp band is comprised of 84 bp and 96 bp bands. Both of these bands co-migrate on a gel.
- Run the products of this master mix separately.

## 7. Assay Procedure

### 7.1. Materials Provided

**Table 3:** Kit components

Gel Detection Kits		ABI Fluorescence Detection Kits	
Catalog #	Description	Catalog #	Description
<b>REF</b> 21010010	IGH Tube A – Unlabeled	<b>REF</b> 21010011	IGH Tube A – 6FAM
<b>REF</b> 21010020	IGH Tube B – Unlabeled	<b>REF</b> 21010101	IGH Tube B – 6FAM
<b>REF</b> 21010030	IGH Tube C – Unlabeled	<b>REF</b> 21010031	IGH Tube C – HEX
<b>REF</b> 21010040	IGH Tube D – Unlabeled	<b>REF</b> 21010041	IGH Tube D – HEX
<b>REF</b> 21010050	IGH Tube E – Unlabeled	<b>REF</b> 21010051	IGH Tube E – 6FAM
<b>REF</b> 20960020	Specimen Control Size Ladder – Unlabeled	<b>REF</b> 20960021	Specimen Control Size Ladder – 6FAM
<b>REF</b> 40881750	IVS-0030 Clonal Control DNA	<b>REF</b> 40881750	IVS-0030 Clonal Control DNA
<b>REF</b> 40881090	IVS-0019 Clonal Control DNA	<b>REF</b> 40881090	IVS-0019 Clonal Control DNA
<b>REF</b> 40881390	IVS-0024 Clonal Control DNA	<b>REF</b> 40881390	IVS-0024 Clonal Control DNA
<b>REF</b> 40880430	IVS-0008 Clonal Control DNA	<b>REF</b> 40880430	IVS-0008 Clonal Control DNA
<b>REF</b> 40920010	IVS-0000 Polyclonal Control DNA	<b>REF</b> 40920010	IVS-0000 Polyclonal Control DNA

### 7.2. Materials Required (not provided)

**Table 3:** Materials Required (not provided)

Reagent/Material	Recommended Reagents/Materials and Suppliers	Catalog Number	Notes
DNA Polymerase	Roche: <ul style="list-style-type: none"> <li>EagleTaq DNA Polymerase</li> </ul> Invivoscribe, Inc. <ul style="list-style-type: none"> <li>FalconTaq DNA Polymerase or equivalent</li> </ul>	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: <ul style="list-style-type: none"> <li>P-2, P-20, P-200, and P-1000 pipettes</li> <li>Or SL-2, SL-20, SL-200, and SL-1000 pipettes</li> </ul>	N/A	Must be able to accurately measure volumes between 1 µL and 1000 µL.
Thermal cycler	Thermo Fisher Scientific: <ul style="list-style-type: none"> <li>Veriti Dx Thermal Cycler</li> </ul> Bio-Rad: <ul style="list-style-type: none"> <li>MJ Research PTC-100 or PTC-200, PTC-220, PTC-240</li> </ul> Perkin-Elmer: <ul style="list-style-type: none"> <li>PE 9600 or PE 9700</li> </ul>	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
<i>Gel Detection</i>			
Ethidium Bromide	Thermo Fisher Scientific: <ul style="list-style-type: none"> <li>UltraPure™ 10 mg/mL Ethidium Bromide</li> </ul>	5585-011	N/A
6% Polyacrylamide Gels	Thermo Fisher Scientific: <ul style="list-style-type: none"> <li>Novex® TBE Gels (6%, 12 well)</li> </ul>	EC62652Box	N/A
TBE Running Buffer	Thermo Fisher Scientific: <ul style="list-style-type: none"> <li>Novex TBE Running Buffer (5X)</li> </ul>	LC6675	Dilute 1:5 prior to use.
Gel Loading Buffer	Thermo Fisher Scientific: <ul style="list-style-type: none"> <li>10X BlueJuice™ Gel Loading Buffer</li> <li>Novex Hi-Density TBE Sample Buffer (5X)</li> </ul>	10816-015 LC6678	N/A

**Table 3:** Materials Required (not provided)

Reagent/Material	Recommended Reagents/Materials and Suppliers	Catalog Number	Notes
100 bp DNA Ladder	Thermo Fisher Scientific: <ul style="list-style-type: none"> <li>TrackIt™ 100 bp DNA Ladder</li> </ul>	10488-058	N/A
<i>ABI Fluorescence Detection</i>			
ABI Capillary Electrophoresis Instrument	Thermo Fisher Scientific: <ul style="list-style-type: none"> <li>ABI 310, 3100, or 3500 series</li> </ul>	N/A	N/A
Hi-Di Formamide	Thermo Fisher Scientific: <ul style="list-style-type: none"> <li>Hi-Di™ Formamide</li> </ul>	4311320	N/A
Size Standards	Invivoscribe, Inc.: <ul style="list-style-type: none"> <li>Hi-Di Formamide w/ROX size standards for ABI 3100</li> </ul> Thermo Fisher Scientific: <ul style="list-style-type: none"> <li>For ABI 3100 or 3130 instruments: <ul style="list-style-type: none"> <li>GeneScan™ - 400HD [ROX]™</li> </ul> </li> <li>For ABI 3500 instruments: <ul style="list-style-type: none"> <li>GeneScan - 600 [LIZ]™ v2.0</li> </ul> </li> </ul>	60980061  402985  4408399	N/A
Spectral Calibration Dye Sets	Thermo Fisher Scientific: <ul style="list-style-type: none"> <li>For ABI 3100 and 3130 instruments: <ul style="list-style-type: none"> <li>DS-30 Matrix Standard Kit (Dye Set D)</li> </ul> </li> <li>For ABI 310 instruments: <ul style="list-style-type: none"> <li>NED Matrix Standard</li> <li>And Fluorescent Amidite Matrix Standards [6FAM, TET, HEX, TAMRA, ROX]</li> </ul> </li> <li>For ABI 3500 instruments: <ul style="list-style-type: none"> <li>DS-33 Matrix Standard Kit (Dye Set G5)</li> </ul> </li> </ul>	4345827  402996  401546  4345833	N/A
Polymer	Thermo Fisher Scientific: <ul style="list-style-type: none"> <li>POP-4™ Polymer: <ul style="list-style-type: none"> <li>POP-4 for 310 Genetic Analyzers</li> <li>POP-4 for 3100/3100-Avant Genetic Analyzers</li> <li>POP-4 for 3130/3130xL Genetic Analyzers</li> </ul> </li> <li>POP-7™ Polymer: <ul style="list-style-type: none"> <li>POP-7 for 3130/3130xL Genetic Analyzers</li> <li>POP-7 for 3500/3500xL Genetic Analyzers</li> </ul> </li> </ul>	402838 4316355 4352755  4352759 4393714	N/A
Buffer	Thermo Fisher Scientific: <ul style="list-style-type: none"> <li>10X Genetic Analyzer Buffer with EDTA</li> </ul>	402824	Dilute 1:10 in sterile water before use

### 7.3. Reagent Preparation

- Unknown samples can be tested using the template amplification control (Specimen Control Size Ladder) master mix to ensure no inhibitors of amplification are present and there is DNA of sufficient quality and quantity to generate a valid result.
- Test all samples in **singlicate**.
- Test positive, negative and no template** controls with 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, 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, then gently vortex to mix.
- 7.3.2. In containment hood or dead air box, remove an appropriate aliquot to clean, sterile microfuge tube (one tube for each master mix).
- 7.3.3. Aliquot volumes = 45 µL for each sample + 135 µL (3 x 45 µL) for the positive, negative and no template controls.
- 7.3.4. Add an additional 20 µL to correct for pipetting errors.
- 7.3.5. Add the appropriate amount of Taq DNA polymerase (0.25 µL @5 U/µL per 50 µL total reaction volume) to each master mix and gently mix by inverting several times or gently vortexing.

## 7.4. Amplification

- 7.4.1. Aliquot 45 µL of the master mix/enzyme solutions into individual PCR wells or tubes.
- 7.4.2. Add 5 µL of sample or control DNA to the individual tubes or wells containing the respective master mix reactions.
- 7.4.3. Pipette up and down several times to mix.
- 7.4.4. Amplify the reactions using the following PCR program:
- 7.4.5. Use the **calculated** option for temperature measurement with the PTC instruments.

**Table 4:** 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

## 7.5. Detection

### Gel Detection – Polyacrylamide TBE Gels

- 7.5.1. Assemble electrophoresis unit using a 6% non-denaturing polyacrylamide TBE gel (made with 1X TBE) and 0.5X TBE running buffer.
- 7.5.2. Add 5 µL of ice-cold non-denaturing bromophenol blue loading buffer to samples.
- 7.5.3. Load 20 µL of mixture into wells of the gel.
- 7.5.4. Run gel at 110V for 2-3 hours or 40-50V overnight.
- 7.5.5. Voltage and electrophoresis time depend on the PCR amplicon size, acrylamide gel thickness and type of PCR equipment.
  - Voltage and run time can be adapted accordingly.
  - Stain gels in 0.5 µg/mL ethidium bromide (in water or 0.5X TBE Buffer) for 5-10 minutes.
- 7.5.6. Destain the gels 2X in water for 5-10 minutes.
- 7.5.7. Visualize the gel using UV illumination.
- 7.5.8. Photograph the gel and interpret the data.

### Gel Detection – Heteroduplex Analysis (RECOMMENDED)

- Note:** Do not heteroduplex the PCR products from the Specimen Control Size Ladder master mix. Skip steps 7.5.9 - 7.5.10 and proceed with step 7.5.11.
- 7.5.9. Denature 20 µL of PCR products at 94°C for 5 minutes.
  - 7.5.10. Re-anneal PCR products at 4°C for 60 minutes.
  - 7.5.11. Assemble electrophoresis unit using a 6% non-denaturing polyacrylamide TBE gel (made with 1X TBE) and 0.5X TBE running buffer.
  - 7.5.12. Add 5 µL of ice-cold non-denaturing bromophenol blue loading buffer to samples.
  - 7.5.13. Load 20 µL of mixture into wells of the gel.
  - 7.5.14. Run gel at 110V for 2-3 hours or 40-50V overnight.
    - Voltage and electrophoresis time depend on the PCR amplicon size, acrylamide gel thickness and type of PCR equipment.
    - Voltage and run time can be adapted accordingly.
  - 7.5.15. Stain gels in 0.5 µg/mL ethidium bromide (in water or 0.5X TBE Buffer) for 5-10 minutes.
  - 7.5.16. Destain the gels 2X in water for 5-10 minutes.
  - 7.5.17. Visualize the gel using UV illumination.
  - 7.5.18. Photograph the gel and interpret the data.

### ABI Fluorescence Detection with ABI 310, 3100 & 3130 instruments

- 7.5.19. In a new microcentrifuge tube, mix an appropriate amount (10 µL per reaction) of Hi-Di Formamide with ROX Size Standards. Vortex well.
- 7.5.20. In a new 96-well PCR plate, add 10 µL of Hi-Di Formamide with ROX size standards to individual wells for each reaction.
- 7.5.21. 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.
- 7.5.22. Cap or cover the PCR plate.
- 7.5.23. Heat denature the samples at 95°C for 2 minutes, then snap chill on ice for 5 minutes.
- 7.5.24. Prepare a sample sheet and injection list for the samples.
- 7.5.25. Run the samples on an ABI 310/3100/3130 capillary electrophoresis instrument according to its user manual.
  - Data are automatically displayed as size and color specific peaks.
- 7.5.26. Review profile and controls, report results.

### ABI Fluorescence Detection with ABI 3500 instruments

- 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.
- 7.5.27. In a new microcentrifuge tube, mix an appropriate amount (9.5 µL per reaction) of Hi-Di Formamide with LIZ Size Standards. Vortex well.
  - 7.5.28. In a new 96-well PCR plate, add 9.5 µL of Hi-Di Formamide with LIZ size standards to individual wells for each reaction.
  - 7.5.29. Transfer 0.5 µL of each reaction to the wells containing Hi-Di Formamide with LIZ size standards.
    - Add only one sample per well.
    - Pipette up and down to mix.
  - 7.5.30. Cap or cover the PCR plate.
  - 7.5.31. Heat denature the samples at 95°C for 3 minutes, then snap chill on ice for 5 minutes.
  - 7.5.32. Prepare a sample sheet and injection list for the samples.
  - 7.5.33. 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.
  - 7.5.34. Review profile and controls, report results.

### 7.6. Quality Control

Positive and negative (or normal) controls are furnished with the kit and can be run in singlicate each time the assay is performed to ensure proper performance of the assay. Include a no template control (*e.g.* water) to test for contamination of the master mix or cross-contamination of PCR. 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 our negative control) are available from Invivoscribe, Inc.

## 7.7. Recommended Positive Controls

The amplicon sizes listed were determined using an ABI platform. Amplicon sizes observed on each specific capillary electrophoresis instrument may differ 1 to 4 base pair (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 each 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 5.** Recommended positive controls

Master Mix	Target	Color	Control DNA	Catalog #	Product Size in base pair (bp)
<i>IGH</i> Tube A	FR1-J <sub>H</sub>	Blue	<b>Valid Size Range</b> IVS-0030 Clonal Control DNA	--- 40881750	<b>310 - 360</b> 280 <sup>a</sup> , 325
<i>IGH</i> Tube B	FR2-J <sub>H</sub>	Blue	<b>Valid Size Range</b> IVS-0030 Clonal Control DNA	--- 40881750	<b>250 – 295<sup>b</sup></b> 260
<i>IGH</i> Tube C	FR3-J <sub>H</sub>	Green	<b>Valid Size Range</b> IVS-0019 Clonal Control DNA	--- 40881090	<b>100 - 170</b> 145
<i>IGH</i> Tube D	D <sub>H</sub> -J <sub>H</sub>	Green	<b>Valid Size Range</b> IVS-0024 Clonal Control DNA	--- 40881390	<b>110 - 290, 390 - 420</b> 139
<i>IGH</i> Tube E	D <sub>H</sub> 7-J <sub>H</sub>	Blue	<b>Valid Size Range</b> IVS-0008 Clonal Control DNA	--- 40880430	<b>100 - 130<sup>d</sup></b> 109
Specimen Control Size Ladder	Multiple Genes	Blue	<b>Valid Size Range</b> IVS-0000 Polyclonal Control DNA	--- 40920010	<b>96, 197, 297, 397, 602<sup>c</sup></b> 96, 197, 297, 397, 602 <sup>c</sup>

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

<sup>b</sup>**Note:** A non-specific peak may be observed at 227 bp.

<sup>c</sup>**Note:** A 209 bp PCR product represents the smallest background peak derived from the germline D<sub>H</sub>7-J<sub>H</sub>1 region. When the PCR amplification is very efficient, longer products might also be obtained because of primer annealing to downstream J<sub>H</sub> gene rearrangements; e.g. 416 bp (D<sub>H</sub>7-J<sub>H</sub>2), 1028nt (D<sub>H</sub>7-J<sub>H</sub>3), etc.

<sup>d</sup>**Note:** Because smaller PCR fragments are preferentially amplified, it is not unusual for the 602 bp fragment to have a diminished signal or to be missing entirely.

## 8. Interpretation of Results

This assay is for research use only. PCR based testing does not identify 100% of clonal cell populations; therefore, repeat testing by Next Generation Sequencing may be advisable to rule out clonality.

### 8.1. Interpretation

- 8.1.1. Report samples that fail to amplify following repeat testing as “A result cannot be reported on this sample because there was DNA of insufficient quantity or quality for analysis”.
- 8.1.2. 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.

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

- **Negative Control:** (Polyclonal control, water or no template blank). If the negative control is:
  - **Positive:** Possible contamination of all PCR amplifications. Do not continue with the interpretation of results. Prepare fresh master mix and repeat amplification.
  - **Negative:** Continue with the analysis.
- **Positive Control:** (This can also be an extraction control if positive control material is taken through extraction processes). If the positive control is:
  - **Positive:** Continue with analysis.
  - **Negative:** Repeat the assay.
- **Specimen Control Size Ladder:** (This is run on unknown samples only). If the amplification control:
  - **Generates 96, 197, 297, 397 and 602 bp products:** Because smaller PCR fragments are preferentially amplified, it is not unusual for the 602 bp fragment to have a diminished signal or to be missing entirely. Continue with analysis.
  - **Does not generate specified products:** Repeat assay and/or re-extract the specimen.

### 8.2. Analysis

Following the acceptance of the controls, the samples are interpreted as follows:

- One or two prominent bands within the valid size range are reported as “Detection of clonal immunoglobulin heavy chain gene rearrangement consistent with the presence of a clonal cell population”.
- Results can be reported as “Positive” or “Negative” for “Detection of clonal immunoglobulin heavy chain gene rearrangement consistent with the presence of a clonal cell population”

## 9. Limitations of Procedure

The assay is subject to interference by degradation of DNA or inhibition of PCR due to heparin or other agents.

## 10. Expected Values

### 10.1. Expected Size of Amplified Products

The size range for each master mix has been determined testing positive control samples. Ignore peaks that occur outside of the valid size range for each master mix. Peaks that are outside of the range cannot be assumed to be valid.

**Note:** “Color” indicates the color of products generated with the master mix when using differential fluorescence detection format (e.g., ABI instruments).

**Table 6.** Expected sizes of amplified products

Master Mix	Target	Color	Control DNA	Catalog #	Product Size in bp
<i>IGH</i> Tube A	FR1-J <sub>H</sub>	Blue	<b>Valid Size Range</b>	---	<b>310 - 360</b>
			IVS-0000 Polyclonal Control DNA	40920010	85, 310 - 360
			IVS-0030 Clonal Control DNA	40881750	280 <sup>a</sup> , 325
			IVS-0019 Clonal Control DNA	40881090	345
			IVS-0024 Clonal Control DNA	40881390	342
<i>IGH</i> Tube B	FR2-J <sub>H</sub>	Blue	<b>Valid Size Range</b>	---	<b>250 – 295<sup>b</sup></b>
			IVS-0000 Polyclonal Control DNA	40920010	250 - 295
			IVS-0030 Clonal Control DNA	40881750	260
			IVS-0019 Clonal Control DNA	40881090	285
			IVS-0024 Clonal Control DNA	40881390	277
<i>IGH</i> Tube C	FR3-J <sub>H</sub>	Green	<b>Valid Size Range</b>	---	<b>100 - 170</b>
			IVS-0000 Polyclonal Control DNA	40920010	100 - 170
			IVS-0019 Clonal Control DNA	40881090	145
<i>IGH</i> Tube D	D <sub>H</sub> -J <sub>H</sub>	Green	<b>Valid Size Range</b>	---	<b>110 - 290, 390 - 420</b>
			IVS-0000 Polyclonal Control DNA	40920010	235 - 258, 344 <sup>c</sup>
			IVS-0030 Clonal Control DNA	40881750	--- <sup>d</sup>
			IVS-0019 Clonal Control DNA	40881090	--- <sup>d</sup>
			IVS-0024 Clonal Control DNA	40881390	139
			IVS-0008 Clonal Control DNA	40880430	--- <sup>d</sup>
<i>IGH</i> Tube E	D <sub>H</sub> 7-J <sub>H</sub>	Blue	<b>Valid Size Range</b>	---	<b>100 - 130</b>
			IVS-0000 Polyclonal Control DNA	40920010	209 <sup>e</sup> , 416 <sup>e</sup>
			IVS-0030 Clonal Control DNA	40881750	--- <sup>f</sup>
			IVS-0019 Clonal Control DNA	40881090	--- <sup>f</sup>
			IVS-0024 Clonal Control DNA	40881390	--- <sup>f</sup>
			IVS-0008 Clonal Control DNA	40880430	109
Specimen Control Size Ladder	Multiple Genes	Blue	<b>Valid Size Range</b>	---	<b>96, 197, 297, 397, 602<sup>g</sup></b>
			IVS-0000 Polyclonal Control DNA	40920010	96, 197, 297, 397, 602 <sup>g</sup>

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

<sup>b</sup>Note: A nonspecific peak may be observed at 227 bp.

<sup>c</sup>Note: A nonspecific 344 bp peak is the result of cross-annealing of the D<sub>H</sub>2 primer to a sequence in the region upstream of J<sub>H</sub>4. In fragment analysis, this peak does not co-migrate with D-J products.

<sup>d</sup>Note: Nonspecific peaks of 74, 94, 158, 176, 187, 200, and 344 bp are observed with many different cell lines. These bands are absent in the presence of polyclonal backgrounds.

<sup>e</sup>Note: A 209 bp PCR product represents the smallest background peak derived from the germline D<sub>H</sub>7-J<sub>H</sub>1 region. When the PCR amplification is efficient, longer products might also be obtained because of primer annealing to downstream J<sub>H</sub> gene rearrangements; e.g. 416 bp (D<sub>H</sub>7-J<sub>H</sub>2), 1028 bp (D<sub>H</sub>7-J<sub>H</sub>3), etc.

<sup>f</sup>Note: Nonspecific peaks of 79 and 123 bp are observed with many different cell lines. These bands are absent in the presence of polyclonal backgrounds.

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



## 10.2. Sample Data

10.2.1. **Gel Detection.** The data shown in Figures 2 - 6 were generated using the master mixes indicated.

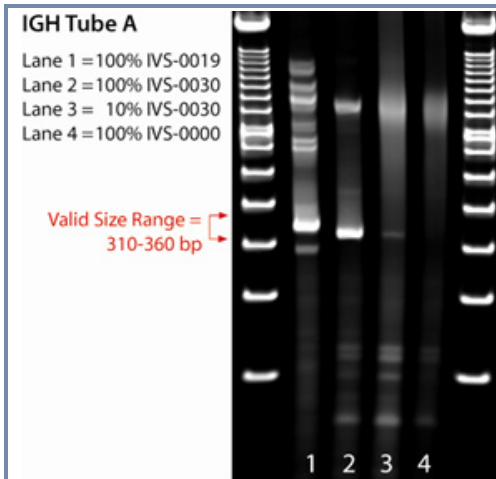


Figure 2. Products amplified with *IGH* Tube A master mix run on a 6% polyacrylamide gel.

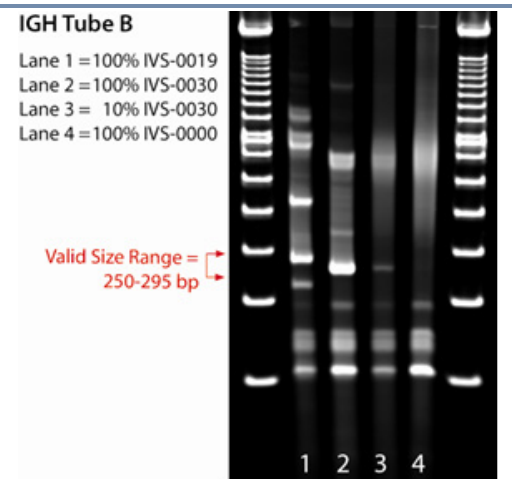


Figure 3. Products amplified with *IGH* Tube B master mix run on a 6% polyacrylamide gel.

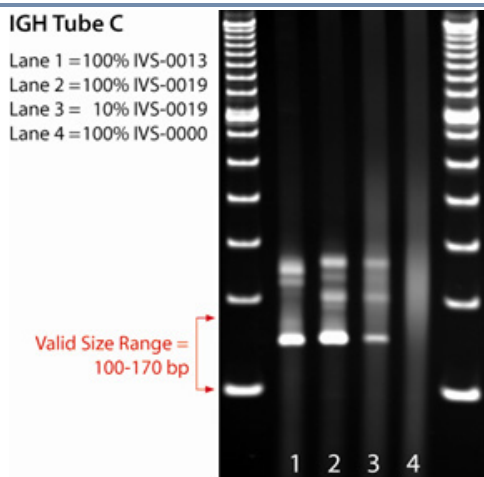


Figure 4. Products amplified with *IGH* Tube C master mix run on a 6% polyacrylamide gel.

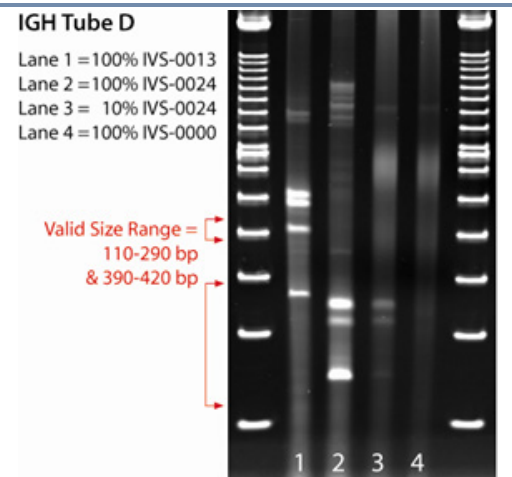


Figure 5. Products amplified with *IGH* Tube D master mix run on a 6% polyacrylamide gel.

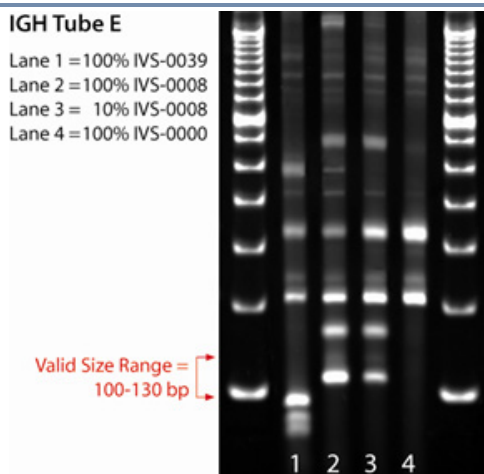
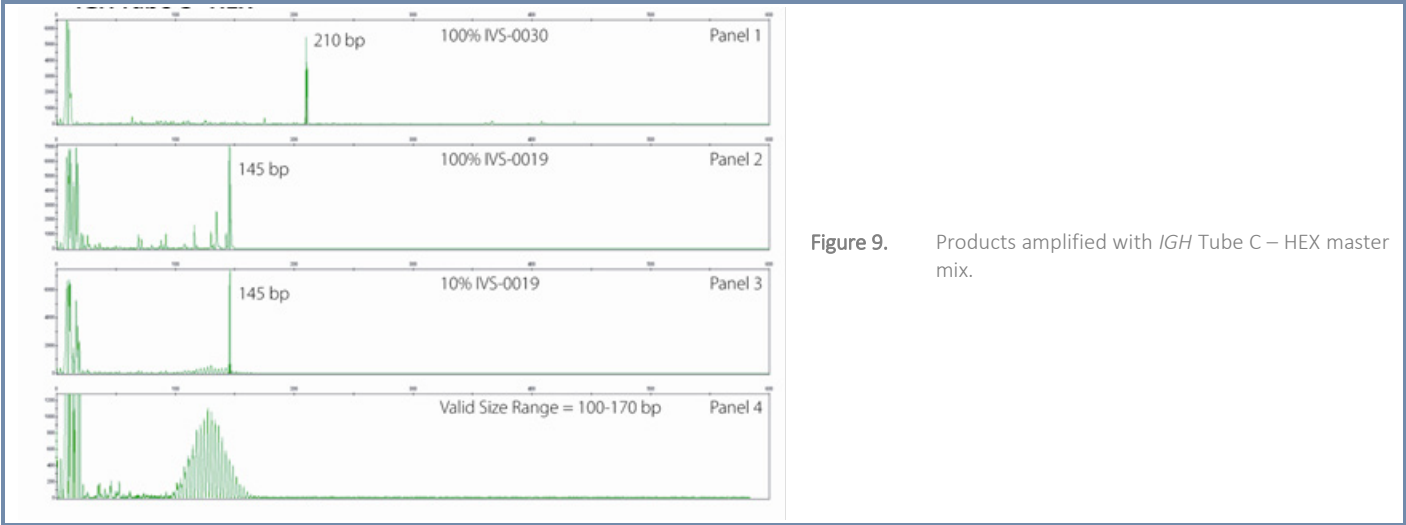
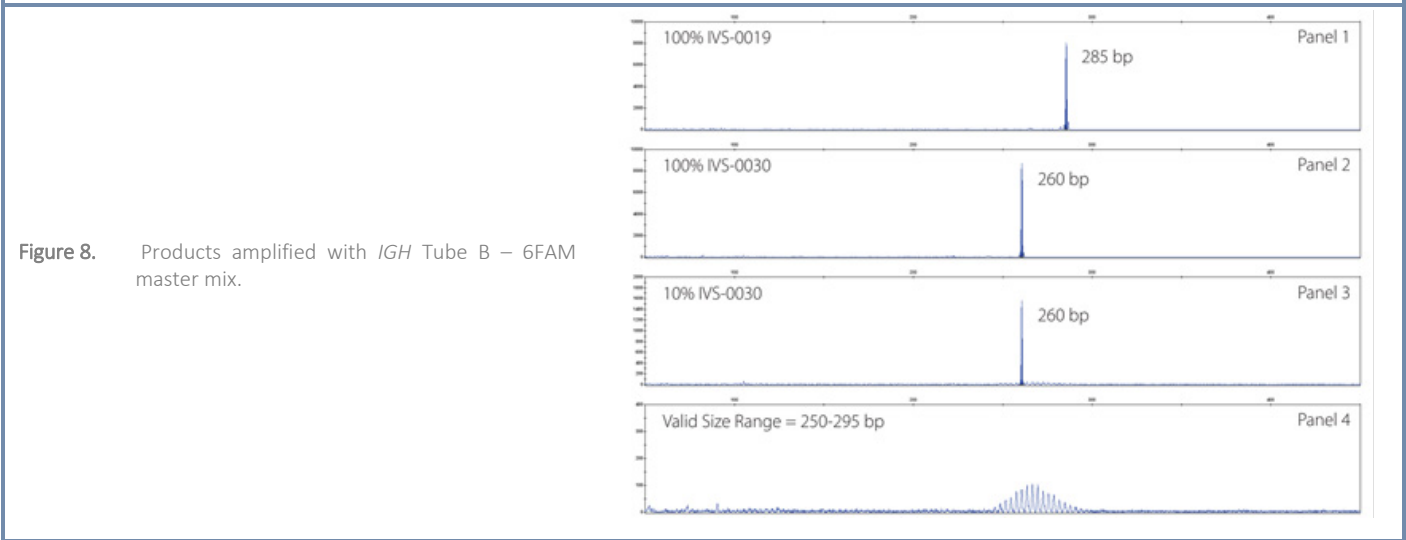
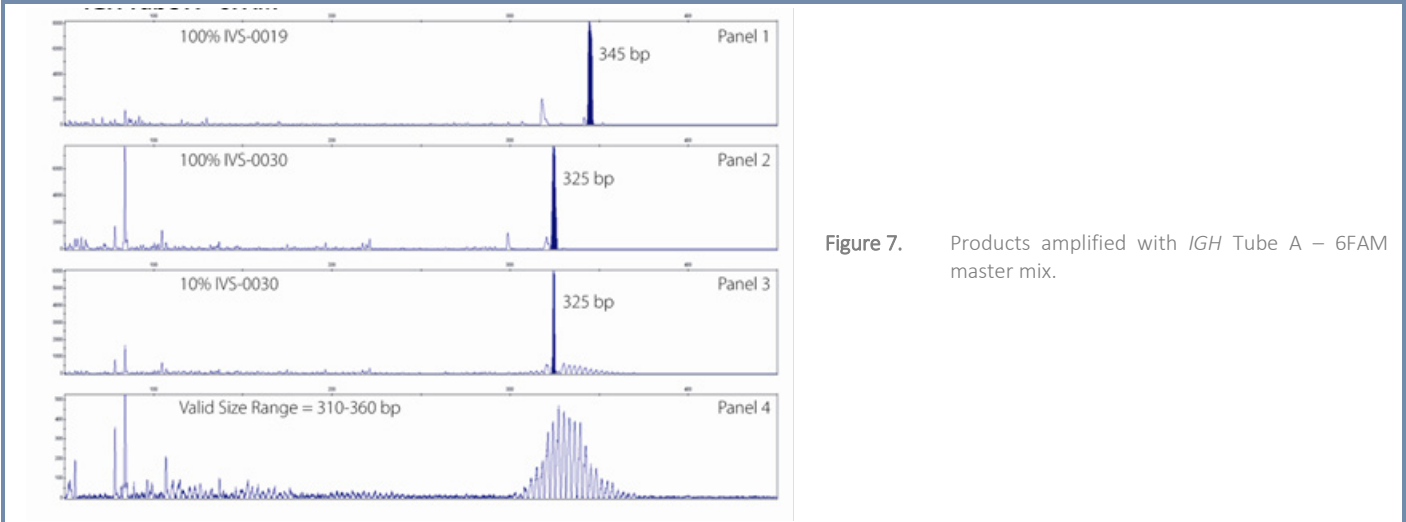
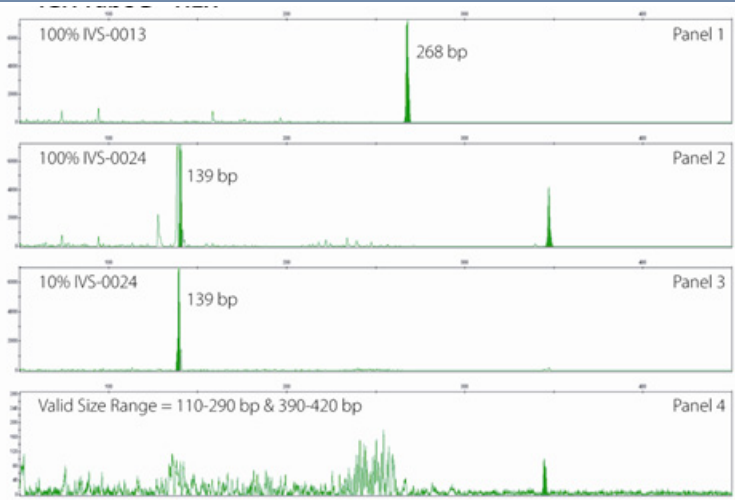


Figure 6. Products amplified with *IGH* Tube E master mix run on a 6% polyacrylamide gel.

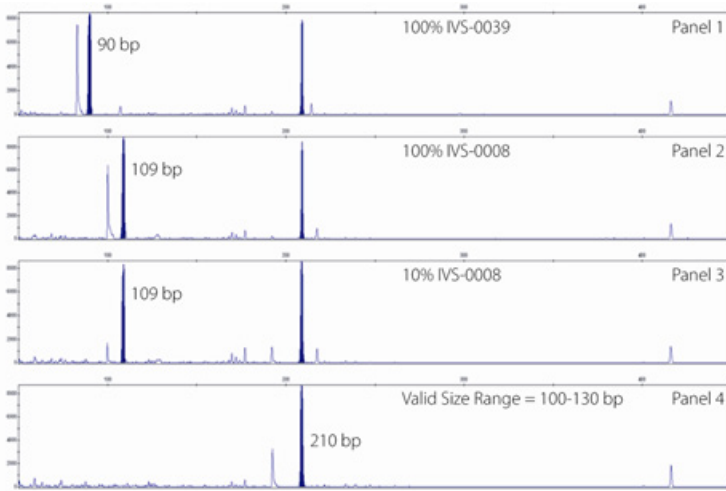
10.2.2. **ABI Fluorescence Detection.** The data shown below (Figures 7 – 11) were generated using the master mixes indicated. Amplified products were run on an ABI instrument.



**Figure 10.** Products amplified with *IGH* Tube D – HEX master mix.



**Figure 11.** Products amplified with *IGH* Tube E – 6FAM master mix.



## 11. Technical and Customer Service

Thank you for purchasing our *IGH* Gene Clonality Assay. We appreciate your business and are happy to assist you in the validation of this assay and will provide ongoing technical assistance.

### Contact Information



Invivoscribe, Inc

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## 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. 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

## 13. Symbols

The following symbols are used in labeling for Invivoscribe products.

	Catalog Number		Expiration Date
	Reagent Volume		Manufacturer
	Lot Number		Consult Instructions for Use
	Storage Conditions		Research Use Only

## 14. Legal Notice

### 14.1. 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 for Research Use Only; not for use in diagnostic procedures.

### 14.2. Associated Patents

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® covered in 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.

## 15. *IGH* Gene Clonality Assay: Single Page Guide

- 15.1. Using gloved hands, remove the master mixes from the freezer. Allow the tubes to thaw; then gently vortex to mix.
- 15.2. In a containment hood or dead air box remove an appropriate aliquot to clean, sterile microfuge tube (one tube for each of the master mixes).
  - Aliquot volumes = 45  $\mu$ L for each sample + 135  $\mu$ L for the positive, negative and no template controls.
  - Add an additional 20  $\mu$ L to correct for pipetting errors.
- 15.3. Add 0.25  $\mu$ L of Taq DNA polymerase @5 U/ $\mu$ L per 50  $\mu$ L total reaction volume to each master mix and gently mix by inverting several times or gently vortexing.
- 15.4. Aliquot 45  $\mu$ L of master mix to individual wells of a PCR plate.
- 15.5. Add 5  $\mu$ L of DNA from the unknown and control samples to individual tubes or wells containing the respective master mix reactions and pipette up and down several times to mix.
- 15.6. Amplify target DNA using the universal thermal cycler program.

### Gel Detection – Heteroduplex Analysis

- 15.7. Denature 20  $\mu$ L of PCR products at 94°C for 5 minutes.
- 15.8. Re-anneal PCR products at 4°C for 60 minutes.
- 15.9. Assemble electrophoresis unit using a 6% non-denaturing polyacrylamide TBE gel and 0.5X TBE running buffer.
- 15.10. Add 5  $\mu$ L of ice-cold non-denaturing bromophenol blue loading buffer to samples
- 15.11. Load 20  $\mu$ L of mixture into wells of the gel.
- 15.12. Run gel at 110V for 2-3 hours or 40-50V overnight.
- 15.13. Stain gels in 0.5  $\mu$ g/mL ethidium bromide (in water or 0.5X TBE Buffer) for 5-10 minutes.
- 15.14. Destain gels 2X in water for 5-10 minutes.
- 15.15. Visualize the gel with UV illumination.
- 15.16. Photograph the gel and interpret the data.

### ABI Fluorescence Detection with ABI 310, 3100 & 3130 instruments

- 15.17. In a new microcentrifuge tube, mix an appropriate amount (10  $\mu$ L per reaction) of Hi-Di Formamide with ROX Size Standards. Vortex well.
- 15.18. In a new 96-well PCR plate, add 10  $\mu$ L of Hi-Di Formamide with ROX size standards to individual wells for each reaction.
- 15.19. Transfer 1.0  $\mu$ L 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.
- 15.20. Cap or cover the PCR plate.
- 15.21. Heat denature the samples at 95°C for 2 minutes, then snap chill on ice for 5 minutes.
- 15.22. Prepare a **sample sheet** and **injection list** for the samples.
- 15.23. Run the samples on an ABI 310 or 3100 capillary electrophoresis instrument according to its user manual.
- 15.24. Review profile and controls, report results.

### ABI Fluorescence Detection with ABI 3500 instruments

- 15.25. In a new microcentrifuge tube, mix an appropriate amount (9.5  $\mu$ L per reaction) of Hi-Di Formamide with LIZ Size Standards. Vortex well.
- 15.26. In a new 96-well PCR plate, add 9.5  $\mu$ L of Hi-Di Formamide with LIZ size standards to individual wells for each reaction.
- 15.27. Transfer 0.5  $\mu$ L of each reaction to the wells containing Hi-Di Formamide with LIZ size standards.
  - Add only one sample per well.
  - Pipette up and down to mix.
- 15.28. Cap or cover the PCR plate.
- 15.29. Heat denature the samples at 95°C for 3 minutes, then snap chill on ice for 5 minutes.
- 15.30. Prepare a **sample sheet** and **injection list** for the samples.
- 15.31. Run the samples on an ABI 3500 capillary electrophoresis instrument according to its user manual.
- 15.32. Review profile and controls, report results.