Instructions for Use

IGH + *IGK* B-Cell Clonality Assay

RUO

For identification of clonal immunoglobulin heavy chain and kappa light chain gene rearrangements.

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

Manufactured in U.S.A.

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 11000010	*IGH* + *IGK* B-Cell Clonality Assay for Gel Detection	33 Reactions
REF 11000031	*IGH* + *IGK* B-Cell Clonality Assay for ABI Fluorescence Detection	33 Reactions
REF 11000041	*IGH* + *IGK* B-Cell Clonality Assay MegaKit for ABI Fluorescence Detection	330 Reactions

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1. Assay Use

Immunoglobulin heavy chain (*IGH*) and Kappa light chain (*IGK*) gene rearrangement assays are 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.[1] 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. *IGH* Tubes A, B, and C target the framework 1, 2, and 3 regions (respectively) within the variable (V_H) region and the joining (J_H) region of the *IGH* locus. *IGK* Tubes A and B target the variable, intragenic and joining regions of the *IGK* locus. 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.

EuroClonality

3. Principles of the Procedure

3.1. Polymerase Chain Reaction (PCR)

Polymerase chain reaction (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) V_H and J_H regions (*IGH* Tubes A, B, and C) of the *IGH* locus, the Vκ and Jκ regions (*IGK* Tube A) and the variable, intragenic and Kappa Deleting Element (K_{de}) regions (*IGK* Tube B) of the of the *IGK* locus. 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 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 this region is amplified using DNA primers that flank this region, a clonal population of cells yields one or two prominent amplified products (amplicons) within the expected size range. Two products are generated in cases when the initial rearrangement was non-productive and was followed by rearrangement of the other homologous chromosome. In contrast, DNA from a normal or polyclonal (many clones) population produces a bell-shaped curve of amplicon products (Gaussian distribution) that reflect the heterogeneous population of V-J region rearrangements.

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 smear of different-sized amplicon products that form a Gaussian distribution around a statistically favored, average-sized rearrangement. As expected, primers that

amplify from the different FR regions, which are located at three distinct regions along the heavy chain gene, produce a correspondingly different size-range of V-J products.

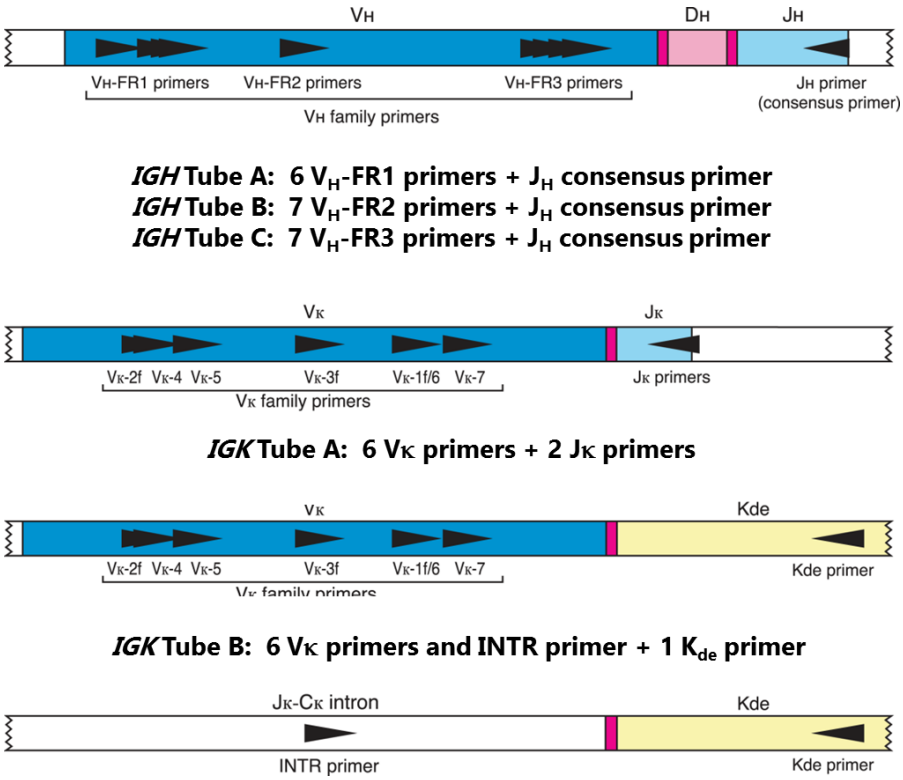

Figure 1. Depicted is a simple representation of the organization of a rearranged immunoglobulin heavy chain *(IGH)* gene on chromosome 14 and the immunoglobulin kappa light chain gene on chromosome 2p11.2. Black arrows represent the relative positions of primers that target the conserved framework (FR1-3) and the downstream consensus J_H gene segments for *IGH* and the VK, JK, 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.

3.2. Gel Electrophoresis

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 which 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.

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*	Quantity
REF 11000010	*IGH* Gene Clonality Assay – Gel Detection	33 Reactions
REF 11000031	*IGH* Gene Clonality Assay – ABI Fluorescence Detection	33 Reactions
REF 11000041	*IGH* Gene Clonality Assay MegaKit – ABI Fluorescence Detection	330 Reactions

Table 2: Kit Components

Reagent	Catalog #	Reagent Components (active ingredients)	Unit Quantity	Assay Kit # of Units	Assay MegaKit # of Units	Storage Temp.
Positive Control DNAs	REF 40881750	**IVS-0030 Clonal Control DNA** 200 µg/mL of DNA in 1/10th TE solution	100 µL	1	5	2°C – 8°C or -85°C – -65°C
	REF 40881090	**IVS-0019 Clonal Control DNA** 200 µg/mL of DNA in 1/10th TE solution	100 µL	1	5	
	REF 40880370	**IVS-0007 Clonal Control DNA** 200 µg/mL of DNA in 1/10th TE solution	100 µL	1	5	
Negative (Normal) Control DNA	REF 40920010	**IVS-0000 Polyclonal Control DNA** 200 µg/mL of DNA in 1/10th TE solution	100 µL	1	5	
Gel Detection						
Master Mixes for Gel Detection	REF 21010010	***IGH* Tube A – Unlabeled** Multiple oligonucleotides targeting the framework 1 region of the immunoglobulin heavy chain gene in a buffered salt solution.	1500 µL	1	N/A	-85°C – -65°C
	REF 21010020	***IGH* Tube B – Unlabeled** Multiple oligonucleotides targeting the framework 2 region of the immunoglobulin heavy chain gene in a buffered salt solution.	1500 µL	1	N/A	
	REF 21010030	***IGH* Tube C – Unlabeled** Multiple oligonucleotides targeting the framework 3 region of the immunoglobulin heavy chain gene in a buffered salt solution.	1500 µL	1	N/A	
	REF 21020010	***IGK* Tube A - Unlabeled** Multiple oligonucleotides targeting the variable and joining regions of the immunoglobulin kappa light chain gene in a buffered salt solution.	1500 µL	1	N/A	
	REF 21020020	***IGK* Tube B - Unlabeled** 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	N/A	
Template Amplification Control Master Mix	REF 20960020	**Specimen Control Size Ladder – Unlabeled** Multiple oligonucleotides targeting housekeeping genes.	1500 µL	1	N/A	
Differential Fluorescence Detection						

Table 2: Kit Components

Reagent	Catalog #	Reagent Components (active ingredients)	Unit Quantity	Assay Kit # of Units	Assay MegaKit # of Units	Storage Temp.
Master Mixes for Differential Fluorescence Detection	REF 21010011	***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
	REF 21010101	***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	
	REF 21010031	***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	
	REF 21020011	***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	
	REF 21020021	***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	REF 20960021	**Specimen Control Size Ladder – 6FAM** Multiple oligonucleotides targeting housekeeping genes.	1500 µL	1	10	

4.2. Warnings and Precautions

- RUO For RESEARCH USE ONLY. Not for use in 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 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, 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 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. 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 your polymer and capillary type.
- See section 7.5 *Detection*.

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 Preparation

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

6.4.2. 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.*, 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.

6.5. Sample Storage

Store samples using a method that prevents degradation of DNA.

6.6. Template Amplification Control

6.6.1. 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

IGH + *IGK* B-Cell Clonality Assay for Gel Detection	
Catalog #	Description
REF 21010010	*IGH* Tube A – Unlabeled
REF 21010020	*IGH* Tube B – Unlabeled
REF 21010030	*IGH* Tube C – Unlabeled
REF 21020010	*IGK* Tube A – Unlabeled
REF 21020020	*IGK* Tube B – Unlabeled
REF 20960020	Specimen Control Size Ladder – Unlabeled
REF 40881750	IVS-0030 Clonal Control DNA
REF 40881090	IVS-0019 Clonal Control DNA
REF 40880370	IVS-0007 Clonal Control DNA
REF 40920010	IVS-0000 Polyclonal Control DNA

IGH + *IGK* B-Cell Clonality Assay for ABI Fluorescence Detection	
Catalog #	Description
REF 21010011	*IGH* Tube A – 6FAM
REF 21010101	*IGH* Tube B – 6FAM
REF 21010031	*IGH* Tube C – HEX
REF 21020011	*IGK* Tube A – 6FAM
REF 21020021	*IGK* Tube B – 6FAM
REF 20960021	Specimen Control Size Ladder – 6FAM
REF 40881750	IVS-0030 Clonal Control DNA
REF 40881090	IVS-0019 Clonal Control DNA
REF 40880370	IVS-0007 Clonal Control DNA
REF 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 Number	Notes
DNA Polymerase	Roche: • EagleTaq DNA Polymerase Invivoscribe: • 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: • MJ Research 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
Gel Detection			

Table 4: Materials Required (not provided)

Reagent/Material	Recommended Reagents/Materials and Suppliers	Catalog Number	Notes
Gel Electrophoresis Unit	N/A	N/A	For polyacrylamide gels
Ethidium Bromide	Thermo Fisher Scientific: • UltraPure™ 10 mg/mL Ethidium Bromide	5585-011	N/A
6% Polyacrylamide Gels	Thermo Fisher Scientific: • Novex® TBE Gels (6%, 12 well)	EC62652Box	N/A
TBE Running Buffer	Thermo Fisher Scientific: • Novex TBE Running Buffer (5X)	LC6675	Dilute 1:5 prior to use.
Gel Loading Buffer	Thermo Fisher Scientific: • 10X BlueJuice™ Gel Loading Buffer • Novex Hi-Density TBE Sample Buffer (5X)	10816-015 LC6678	N/A
100 bp DNA Ladder	Thermo Fisher Scientific: • TrackIt™ 100 bp DNA Ladder	10488-058	N/A
	Differential Fluorescence Detection		
ABI Capillary Electrophoresis Instrument	Thermo Fisher Scientific: • ABI 310, 3100, or 3500 series	N/A	N/A
Hi-Di Formamide	Thermo Fisher Scientific: • Hi-Di™ Formamide	4311320	N/A

Size Standards	Invivoscribe:
	• Hi-Di Formamide w/ROX size standards for ABI 310
	• Hi-Di Formamide w/ROX size standards for ABI 3100
	Thermo Fisher Scientific:
	• For ABI 3100 or 3130 instruments:
	  ○ GeneScan™ - 400HD [ROX]™
	• For ABI 3500 instruments:
	  ○ GeneScan - 600 [LIZ]™ v2.0
		60980051
		60980061
		
		402985
		
		4408399
			N/A

Spectral Calibration Dye Sets	Thermo Fisher Scientific:
	• 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]
	• For ABI 3500 instruments:
	  ○ DS-33 Matrix Standard Kit (Dye Set G5)
		4345827
		
		402996
		
		401546
		
		4345833
			Dye set used to spectrally calibrate ABI instrument for use with 6FAM, HEX, NED, and ROX

Polymer	Thermo Fisher Scientific:
	• 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	Thermo Fisher Scientific: • 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 to ensure that 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 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, 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).

- Aliquot volumes = 45 µL for each sample + 135 µL (3 x 45 µL) for the positive, negative and no template controls.
- Include an additional 20 µL to correct for pipetting errors.

7.3.3. Add the appropriate amount of Taq DNA polymerase (0.25 µL of @5 U/µL per 50 µL total reaction volume) to each master mix and gently mix by inverting several times or gently vortexing.

- The master mixes are now ready for distribution to reaction tubes or plate and addition of sample.

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.

- Pipette up and down several times to mix.

7.4.3. Amplify the reactions using the following PCR program.

- Use the **calculated** option for temperature measurement with the PTC instruments.

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

7.4.4. Remove the amplification plate from the thermal cycler.

7.5. Detection

- Not all detection formats are available for all assays.

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.

- 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.

7.5.5. Stain gels in 0.5 µg/mL ethidium bromide (in water or 0.5X TBE Buffer) for 5-10 minutes.

7.5.6. Destain 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)

- 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 94ºC for 5 minutes.

7.5.10. Re-anneal PCR products at 4ºC 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 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 PCR 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 95ºC 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 PCR 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 PCR reaction.

7.5.29. Transfer 0.5 µL of each PCR reaction to the wells containing Hi-Di Formamide with LIZ size standards. Add only one sample per well. Pipette up and down to mix.

7.5.30. Cap or cover the PCR plate.

7.5.31. Heat denature the samples at 95ºC 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.

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

Table 6: Recommended Positive Controls

Master Mix	Target	Color	Control DNA	Catalog #	Product Size in (bp)
***IGH* Tube A**	FR1-J_H	Blue	**Valid Size Range** IVS-0030 Clonal Control DNA	--- 40881750	**310 - 360** 280[1], 325
***IGH* Tube B**	FR2-J_H	Blue	**Valid Size Range** IVS-0030 Clonal Control DNA	--- 40881750	**250 - 295[2]** 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	**84, 96, 197, 297, 397, 602[3]** 84, 96, 197, 297, 397, 602

Note: The amplicon sizes listed above were determined using an ABI platform. Amplicon sizes seen on your specific CE instrument may differ 1-4 bp from those listed above depending on the platform of detection (ABI) 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.

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

[2]**Note**: A non-specific peak may be observed at 227 bp.

[3]**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 and Reporting

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. Sample Reporting

Results can be reported as "**Positive**" or "**Negative**" for "**Detection of clonal immunoglobulin heavy chain or kappa light chain gene rearrangement consistent with the presence of a clonal cell population**".

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. 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 amplification reactions. 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. Sample Interpretation

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

8.2.1. One or two prominent bands within the valid size range for *IGH* Tubes A, B, or C is reported as "**Detection of clonal immunoglobulin heavy chain gene rearrangement consistent with the presence of a clonal cell population**".

8.2.2. One or two prominent bands within the valid size range for *IGK* Tubes A or B is reported as "**Detection of clonal immunoglobulin kappa light 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 of the master mixes has been determined testing positive control samples. For accurate and meaningful interpretation it is important to ignore peaks that occur outside of the proscribed/valid size range for each of the master mixes. 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 7: Expected size of Amplified Products

Master Mix	Target	Color	Control DNA	Catalog Number	Product Size (bp)
***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** 85, 310 - 360 280[1], 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[2]** 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}	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[3] 274, 282
Specimen Control Size Ladder	Multiple Genes	Blue	Any Human DNA	---	84, 96, 197, 297, 397, 602

Note: The amplicon sizes listed above were determined using an ABI platform. Amplicon sizes seen on your specific CE instrument may differ 1-4 bp from those listed above 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.

[1]Note: The 280 bp band is a known amplicon that lies just outside the valid size range for Tube A.

[2]Note: A non-specific peak may be observed at 227 bp.

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

10.2. Sample Data

Gel Detection

The data shown in Figures 2 -7 were generated using the master mixes indicated:

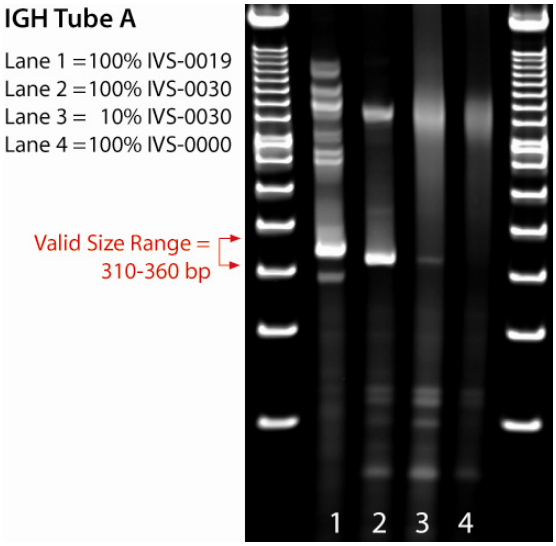

Figure 2. The data shown above was generated using the *IGH* Tube A master mix and run on a 6% polyacrylamide gel.

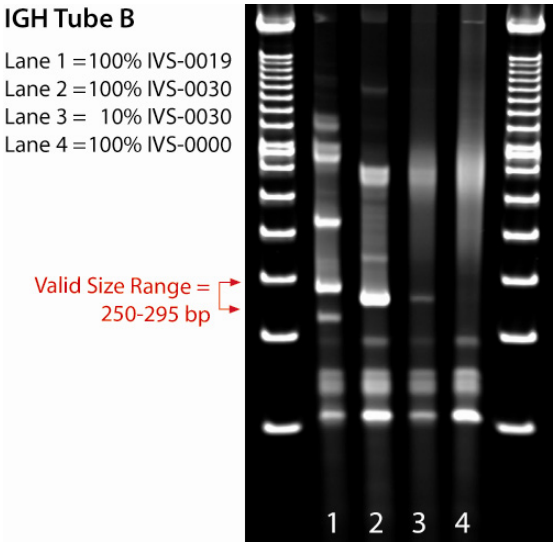

Figure 3. The data shown above was generated using the *IGH* Tube B master mix and run on a 6% polyacrylamide gel.

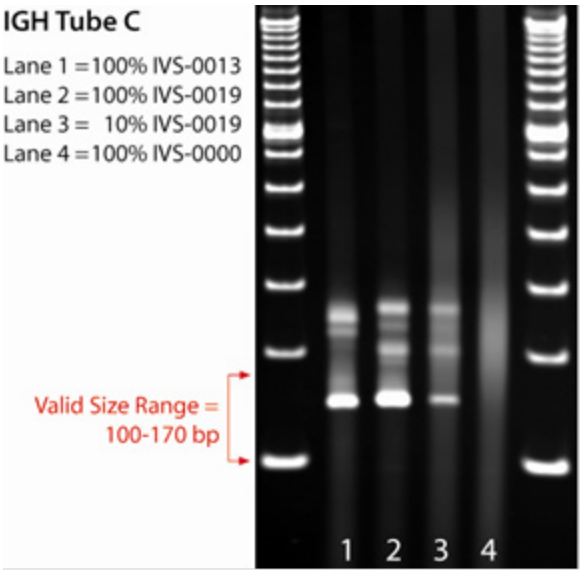

Figure 4. The data shown above was generated using the *IGH* Tube C master mix and run on a 6% polyacrylamide gel.

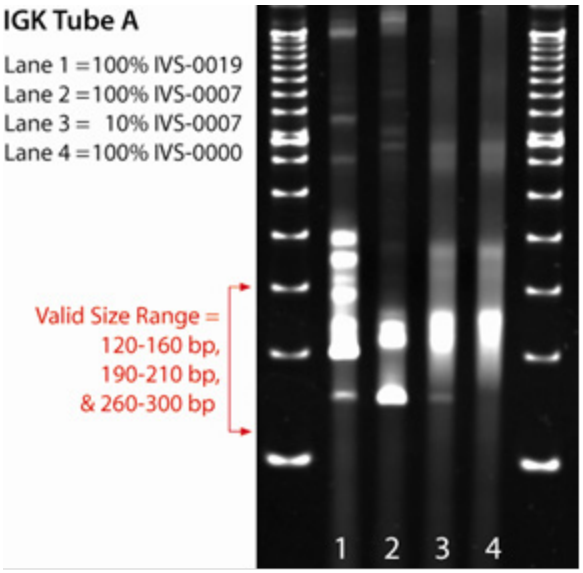

Figure 5. The data shown above was generated using the *IGK* Tube A master mix and run on a 6% polyacrylamide gel.

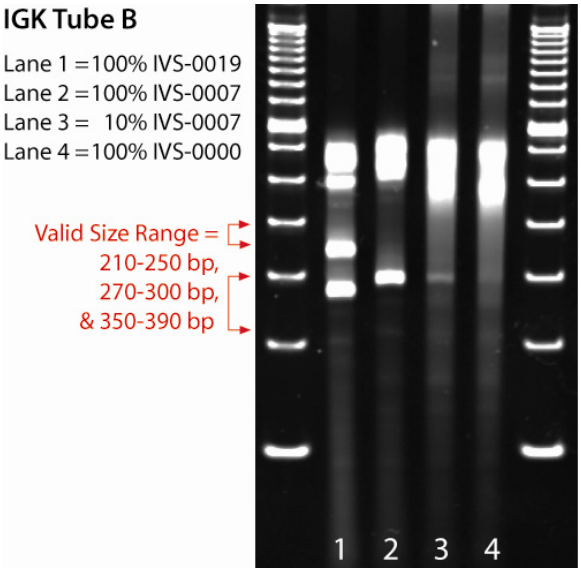

Figure 6. The data shown above was generated using the *IGK* Tube B master mix and run on a 6% polyacrylamide gel.

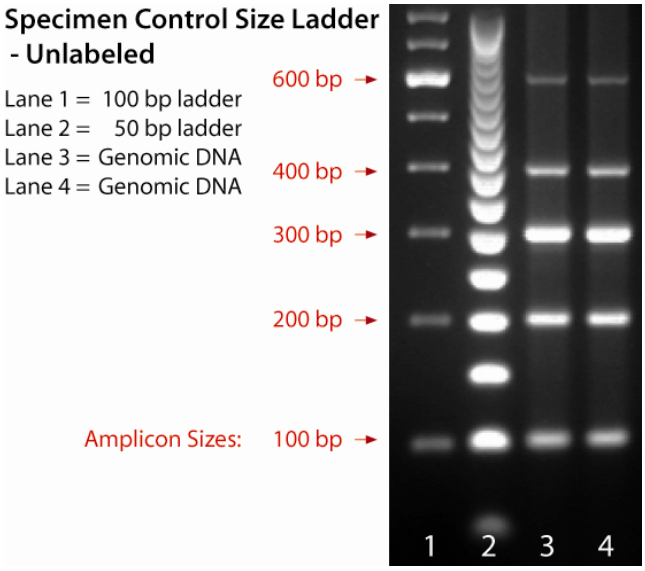

Figure 7. The data shown above was generated using the Specimen Size Control Ladder master mix and run on a 6% polyacrylamide gel.

ABI Fluorescence Detection

The data shown in Figures 8 - 12 were run on an ABI instrument using the master mixes indicated..

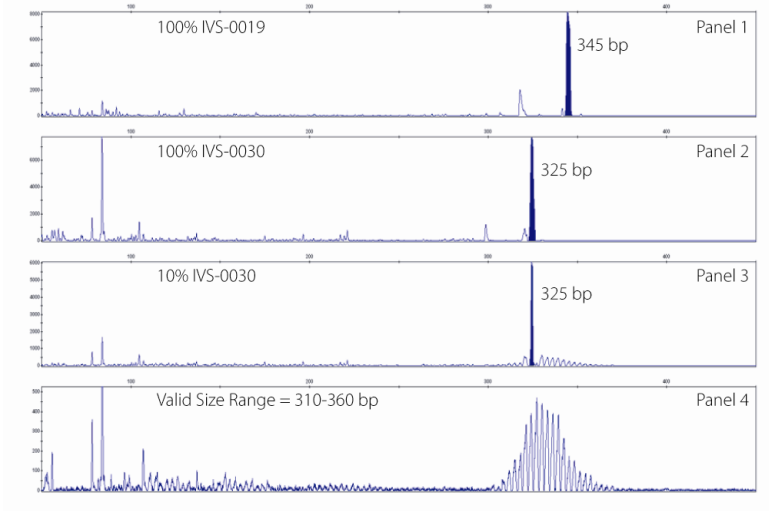

Figure 8. The data shown above was generated using the *IGH* Tube A – 6FAM master mix.

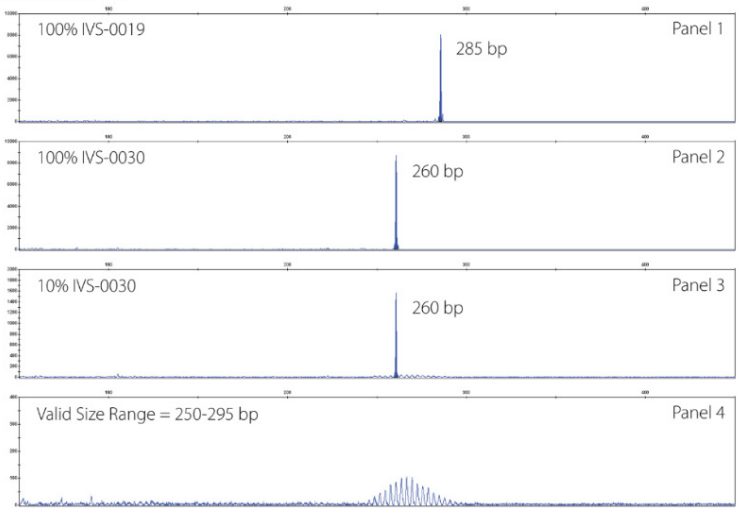

Figure 9. The data shown above was generated using the *IGH* Tube B– 6FAM master mix.

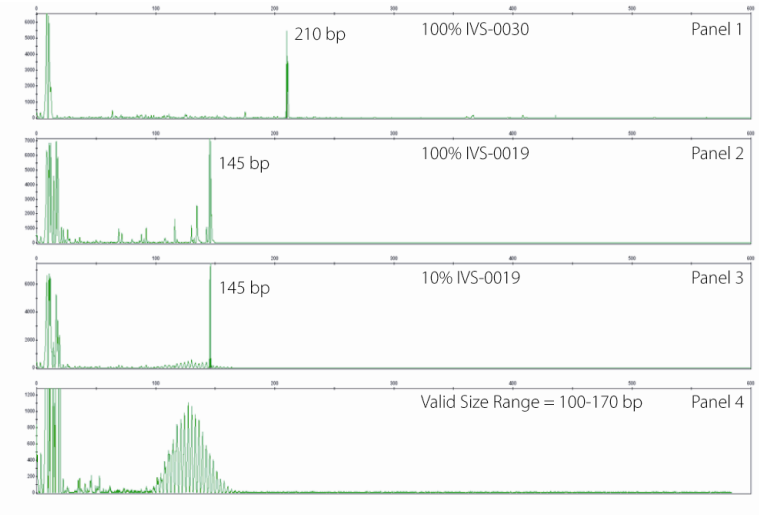

Figure 10. The data shown above was generated using the *IGH* Tube C– HEX master mix.

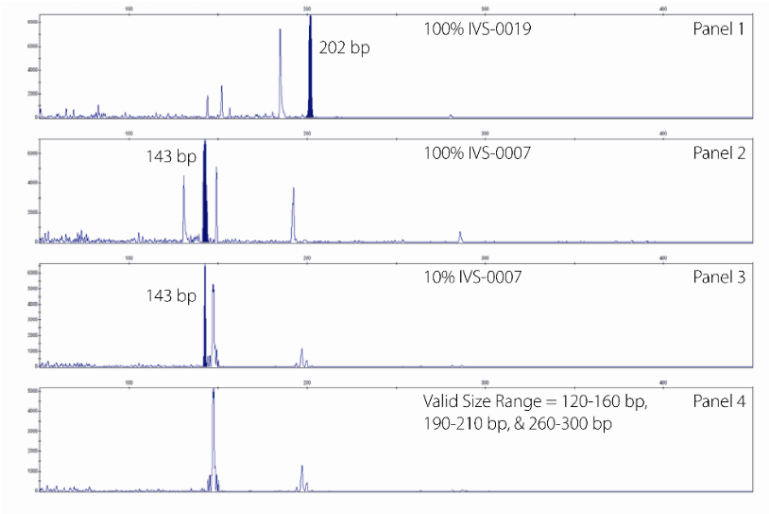

Figure 11. The data shown above was generated using the *IGK* Tube A– 6FAM master mix.

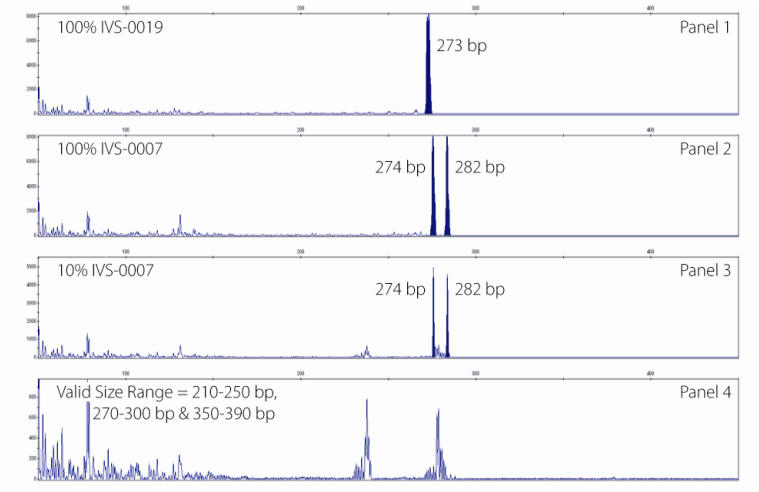

Figure 12. The data shown above was generated using the *IGK* Tube B– 6FAM master mix.

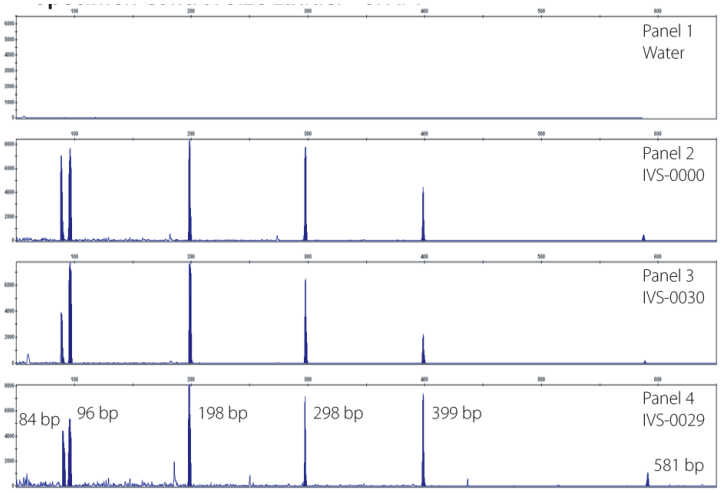

Figure 13. The data shown above was generated using the Specimen Control Size Ladder– 6FAM master mix.

11. Technical and Customer Service

Thank you for purchasing our ***IGH* + *IGK* B-Cell Clonality Assay**. We appreciate your business. We are happy to assist you in the validation of this assay and will provide ongoing technical assistance to keep the assays performing efficiently in your laboratory. Technical assistance is most rapidly obtained using our Internet site: http://www.invivoscribe.com or by sending an email inquiry to: support@invivoscribe.com.

Contact Information

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12. References

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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.
2. 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.

REF	Catalog Number		Expiration Date
VOL	Reagent Volume		Manufacturer
LOT	Lot Number		Consult Instructions for Use
	Storage Conditions	RUO	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.

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 + IGK* B-Cell 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 µL for each sample + 135 µL for the positive, negative and no template controls.
- Add an additional 20 µL to correct for pipetting errors.

15.3. Add the appropriate amount of Taq DNA polymerase (0.25 µL of Taq @5 U/µL per 50 µL total reaction volume) to each master mix and gently mix by inverting several times or gently vortexing.

15.4. Aliquot 45 µL of master mix to individual wells of a PCR plate.

15.5. Add 5 µL 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 µL PCR products at 94ºC for 5 minutes.

15.8. Re-anneal PCR products at 4ºC for 60 minutes.

15.9. Assemble electrophoresis unit using a 6% non-denaturing polyacrylamide TBE gel (made with 1X TBE) and 0.5X TBE running buffer.

15.10. Add 5 µL ice-cold non-denaturing bromophenol blue loading buffer to samples

15.11. Load 20 µL 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 µg/mL ethidium bromide (in water or 0.5X TBE Buffer) for 5-10 minutes.

15.13. Destain gels 2X in water for 5-10 minutes.

15.14. Use UV illumination to visualize gel.

15.15. Photograph the gel and interpret the data.

ABI Fluorescence Detection with ABI 310, 3100 & 3130 instruments

15.16. In a new microcentrifuge tube, mix an appropriate amount (10 µL per reaction) of Hi-Di Formamide with ROX Size Standards. Vortex well.

15.17. In a new 96-well PCR plate, add 10 µL Hi-Di Formamide with ROX size standards to individual wells for each reaction.

15.18. 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.

15.19. Cap or cover the PCR plate.

15.20. Heat denature the samples at 95 ºC for 2 minutes, then snap chill on ice for 5 minutes.

15.21. Prepare a **sample sheet** and **injection list** for the samples.

15.22. Run the samples on an ABI 310/3100/3130 capillary electrophoresis instrument according to its user manual.

15.23. 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.

15.24. In a new microcentrifuge tube, mix an appropriate amount (9.5 µL per reaction) Hi-Di Formamide with LIZ Size Standards. Vortex well.

15.25. In a new 96-well PCR plate, add 9.5 µL Hi-Di Formamide with LIZ size standards to individual wells for each reaction.

15.26. 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.

15.27. Cap or cover the PCR plate.

15.28. Heat denature the samples at 95ºC for 3 minutes, then snap chill on ice for 5 minutes.

15.29. Prepare a sample sheet and injection list for the samples.

15.30. Run the samples on an ABI 3500 capillary electrophoresis instrument according to its user manual.

15.31. Review profile and controls, report results.