Instructions for Use

IGH Somatic Hypermutation Assay v2.0

RUO

To sequence and identify mutational status of clonal *IGH* 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	51010030	*IGH* Somatic Hypermutation Assay v2.0 for Gel Detection	33 Reactions
REF	51010031	*IGH* Somatic Hypermutation Assay v2.0 for ABI Fluorescence Detection	33 Reactions
REF	51010040	*IGH* Somatic Hypermutation Assay MegaKit v2.0 for Gel Detection	330 Reactions
REF	51010041	*IGH* Somatic Hypermutation Assay MegaKit v2.0 for ABI Fluorescence Detection	330 Reactions

Table of Contents

1. Assay Use

The Research Use Only *IGH* Somatic Hypermutation Assay v2.0 is used to identify clonal rearrangements of the immunoglobulin heavy (*IGH*) chain gene and determine the somatic mutation status of the variable (V) gene sequence and is useful for the study of:

- Identifying clonal rearrangements of the IGH chain gene
- Assessing the extent of somatic hypermutation in the variable region of the immunoglobulin heavy chain gene
- Evaluating 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 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-based assay employs multiple consensus DNA primers that target conserved genetic regions within the immunoglobulin heavy chain (*IGH*) gene. This test is used to detect and sequence the majority of clonal *IGH* rearrangements from either genomic DNA (gDNA) or complementary DNA (cDNA). Clonal products can be detected using a variety of methods, including gel and capillary electrophoresis.

The primers that target the leader (VHL) and framework 1 (FR1) regions have been designed to include a universal sequencing tag at the 5'end. This design allows for bi-directional sequencing of clonal PCR products with just one sequencing-tag specific forward primer and one J_H reverse primer. The presence of *IGH* somatic hypermutation (SHM) is defined as greater or equal to 2% difference from the germline variable (V) gene sequence, whereas less than 2% difference is considered evidence of no somatic hypermutation.

2.2. Summary

This test amplifies either gDNA or cDNA that lies between the upstream leader (VHL) or framework 1 (FR1) regions and the downstream joining (J) region of the *IGH* gene. The test utilizes two (2) different master mixes: Hypermutation Mix 1 v2.0 and Hypermutation Mix 2 v2.0. The Hypermutation Mix 1 v2.0 targets sequences between the leader and joining regions. Therefore the amplicon product(s) span the entire variable (V) region, which contains the FR1, CDR1 (complementarity-determining region 1), FR2, CDR2, FR3 and CDR3 regions. The Hypermutation Mix 2 v2.0 targets sequences between the framework 1 (FR1) and joining (J) regions. The resulting amplicons include a portion of the FR1 region to the downstream J region. Accordingly products do not include the complete FR1 sequence.

3. Principles of the Procedure

3.1. Polymerase Chain Reaction (PCR)

PCR assays are routinely used for the identification of clonal lymphocyte populations. Each B-cell has a single productive *IGH* gene rearrangement (consisting of the combination of a variable (V) region, a diversity (D) region and a joining (J) region) that is unique in both length and sequence. Therefore, when gDNA or cDNA from a normal or polyclonal population is amplified using primers that flank the V-J region, a bell-shaped curve (Gaussian distribution) of amplicon products within an expected size range is produced. On a gel, this distribution of products is seen as a smear. This Gaussian distribution reflects the heterogeneous population of V-D-J rearrangements. In cases where lymphocytes are not present, no product is seen. For gDNA or cDNA from samples containing a clonal population, the yield is one or two prominent amplified products (amplicons) within a diminished polyclonal background. Two products are produced in cases where the initial rearrangement was non-productive and was followed by rearrangement of the other homologous chromosome.

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 primer sequences to target all of the conserved flanking regions around the V-D-J rearrangement. N-region diversity and somatic mutation further diversify the genetic sequences in

these regions. Therefore multiplex master mixes, which target several regions such as the leader (L) or framework (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. As expected, primers that amplify from the L or FR regions, produce a correspondingly different size-range of V-D-J products.

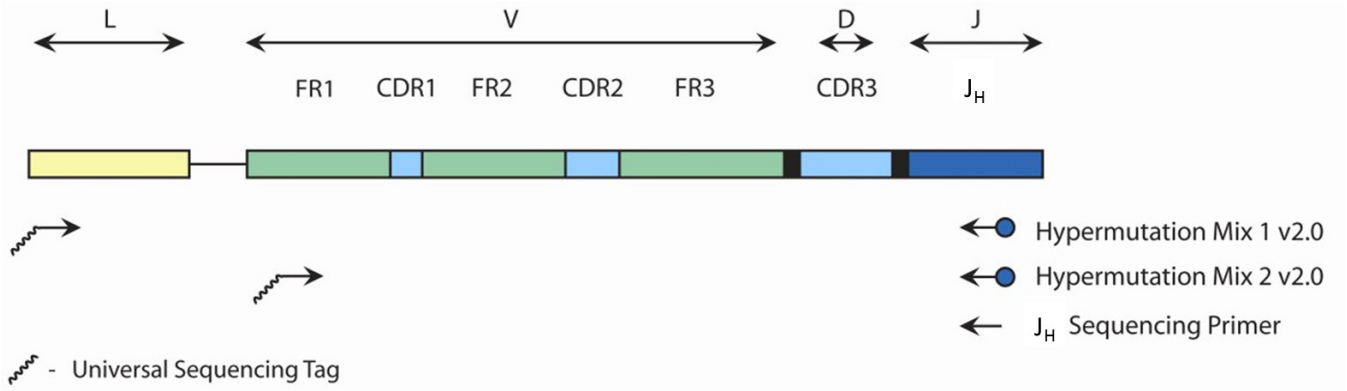

Figure 1. Depicted is a simple representation of the organization of a rearranged immunoglobulin heavy chain gene on chromosome 14. Black arrows represent the relative positions of primers that target the conserved Leader (L) and Framework 1 (FR1) regions, and the downstream consensus J_H gene segments.

3.2. Gel Electrophoresis Detection

Gel electrophoresis, such as agarose gel electrophoresis or non-denaturing polyacrylamide gel electrophoresisis (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 dye 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 separate 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

Differential fluorescence detection is commonly used to resolve the different-sized amplicon products using a capillary electrophoresis instrument. Primers can be conjugated with several different fluorescent dyes (fluorophores) so that they can produce different emission spectra upon excitation by a laser in the capillary electrophoresis instrument. In this manner, different fluorescent dyes can correspond to different targeted regions. This detection system results in unsurpassed sensitivity, single nucleotide resolution, differential product detection and relative quantification. In addition, the use of agarose and polyacrylamide gels, as well as the use of carcinogens such as ethidium bromide, can virtually be eliminated. Further, differential detection allows accurate, reproducible and objective interpretation of primer-specific products and automatic archiving of data. Inter-assay and intra-assay reproducibility in size determination using capillary electrophoresis is approximately 1 to 2 nucleotides.

3.4. *IGH* Somatic Hypermutation (SHM) Analysis

Clonal PCR products are identified and are gel extracted and sequenced. Analysis for somatic hypermutation (SHM) includes sequencing of the full variable region (FR1-FR3) or a partial variable region (CDR1-FR3) to determine mutational status. Mutational status is determined by comparing the sequence of the *IGH*V region of the sample to the most homologous germline V sequence. Sequences that differ by more than 2% from their corresponding germline sequences are considered highly mutated whereas sequences that differ by less than 2% are considered unmutated.[4] Listed below are several websites available to aid in *IGH* Somatic Hypermutation Analyses:

- V BASE – The MRC Centre for Protein Engineering's Database of human antibody genes
  - http://www.vbase2.org
  - Analysis tools: DNAPLOT
- NCBI – National Center for Biotechnology Information
  - http://www.ncbi.nlm.nih.gov/igblast/
  - Analysis tools: IgBLAST (Basic Local Alignment Search Tool)

4. Reagents

4.1. Reagent Components

Table 1. Available Kits

Catalog #	Description	Total Reactions
REF 51010030	*IGH* Somatic Hypermutation Assay v2.0 - Gel Detection	33 Reactions
REF 51010031	*IGH* Somatic Hypermutation Assay v2.0 - ABI Fluorescence Detection	33 Reactions
REF 51010040	*IGH* Somatic Hypermutation Assay MegaKit v2.0 - Gel Detection	330 Reactions
REF 51010041	*IGH* Somatic Hypermutation Assay MegaKit v2.0 - ABI Fluorescence Detection	330 Reactions

Table 2. Reagent Components

Reagent	Catalog # (REF)	Reagent Components (active ingredients)	Unit Quantity	Assay Kit # of Units	Assay MegaKit # of Units	Storage Temp.
Positive Control DNA and RNA	40891090	**IVS-0013 Clonal Control RNA** 400 µg/mL of RNA in water.	100 µL	1	5	-85°C to -65°C
	40880730	**IVS-0013 Clonal Control DNA** 200 µg/mL of DNA in 1/10th TE solution.	100 µL	1	5	2°C to 8°C or -85°C to -65°C
Negative (Normal) Control DNA	40920010	**IVS-0000 Polyclonal Control DNA** 200 µg/mL of DNA in 1/10th TE solution.	100 µL	1	5	
Sequencing Primers	31010380	***IGH* J_H Primer – Unlabeled** 100 µM of DNA primer in water.	10 µL	1	5	-25°C to -15°C or -85°C to -65°C
Sequencing Primers	30000000	**Primer - Hypermutation – Unlabeled** 100 µM of DNA primer in water.	10 µL	1	5	
Differential Fluorescence Detection						
Master Mixes	21010171	**Hypermutation Mix 1 v2.0 – 6FAM** Multiple oligonucleotides targeting the leader region of the immunoglobulin heavy chain gene in a buffered salt solution.	1500 µL	1	10	-85°C to -65°C
	21010181	**Hypermutation Mix 2 v2.0 – 6FAM** Multiple oligonucleotides targeting the framework 1 region of the immunoglobulin heavy chain gene in a buffered salt solution.	1500 µL	1	10	
Template Amplification Control Master Mix	20960021	**Specimen Control Size Ladder – 6FAM** Multiple oligonucleotides targeting housekeeping genes.	1500 µL	1	10	
Gel Detection						
Master Mixes	21010170	**Hypermutation Mix 1 v2.0 - Unlabeled** Multiple oligonucleotides targeting the leader region of the immunoglobulin heavy chain gene in a buffered salt solution.	1500 µL	1	10	-85°C to -65°C
	21010180	**Hypermutation Mix 2 v2.0 - Unlabeled** Multiple oligonucleotides targeting the framework 1 region of the immunoglobulin heavy chain gene in a buffered salt solution.	1500 µL	1	10	
Template Amplification Control Master Mix	20960020	**Specimen Control Size Ladder - Unlabeled** Multiple oligonucleotides targeting housekeeping genes.	1500 µL	1	10	

Note: There are no preservatives used during the manufacturing process of this kit.

4.2. Warnings and Precaution

- **RUO** This product is For Research Use Only
- Use the 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.
- Adherence to the protocol will assure optimal performance and reproducibility. 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 all 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 work flow in the PCR laboratory between separate work areas; begin with Master Mix preparation, move to specimen preparation, then to the amplification and finally to detection. Do not bring amplified DNA into the areas designated for master mix or specimen preparation.
- 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 for long term storage DNA controls can be stored at -85°C to -65°C.
- All reagents and controls must be thawed and vortexed or mixed thoroughly prior to use to ensure that they are resuspended completely. Excessive vortexing may shear DNA and cause labeled primers to lose their fluorophores.
- Materials are stable until the labeled expiration date when stored and handle 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
- 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. Agarose or Polyacrylamide Gel Electrophoresis Unit

- Use or function: DNA fragment separation
- Performance Characteristics and Specification:
  - Capable of maintaining 35-135 V for extended times
- Follow manufacturer's installation, operation, calibration and maintenance procedures.
- See section 7.5 *Detection* for more details.

5.3. UV Illuminator 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 310, ABI 3100 or ABI 3130 (For ABI Fluorescence Detection Assays)

- Use or function: Fragment detection and analysis
- Performance characteristics and Specification:
  - The following capillary electrophoresis instruments will meet the performance needs for this assay:
    - ABI 310 Genetic Analyzer (1-capillary)
    - ABI 3100 Avant Genetic Analyzer (4-capillaries)
    - ABI 3100 Genetic Analyzer (16-capillaries)
    - ABI 3130 Genetic Analyzer (4-capillaries)
    - ABI 3130xL Genetic Analyzer (16-capillaries)
- Follow manufacturer's installation, operation, calibration and maintenance procedures.
- Calibrate the ABI instrument with the Dye Set(s) for 6-FAM, HEX, NED, and ROX.
- Use the default settings for your polymer and capillary type.
- See section 7.5 *Detection* for more 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 gDNA or cDNA derived from RNA. Common sources of gDNA and RNA include:

- 5 cc of peripheral blood, bone marrow biopsy or bone marrow aspirate anti-coagulated with heparin or EDTA (stored at 2ºC to 8ºC and shipped at ambient temperature)
- Formalin-fixed paraffin embedded tissue or slides (stored and shipped at ambient temperature)

6.4. gDNA Sample Preparation

This assay can be used with either gDNA or cDNA.

6.4.1. Extract the gDNA from specimens as soon as possible.

6.4.2. Resuspend DNA to a final concentration of 100 µg to 400 µg per mL in 1/10th TE (1 mM Tris-HCl, pH 8.0; 0.1 mM EDTA) or in molecular biology grade or USP water.

- This is a robust assay system; a wide range of DNA concentrations will generate a valid result.
- Therefore, quantifying and adjusting DNA concentrations is generally not necessary.
- Testing sample DNA with the Specimen Control Size Ladder master mix will ensure that DNA of sufficient quality and quantity was present to yield a valid result.

6.5. gDNA Sample Storage

Store gDNA at 2°C to 8°C or at -85°C to -65°C for long term storage.

6.6. RNA and cDNA Sample Preparation

This assay can be used with either gDNA or cDNA.

6.6.1. Extract total RNA or mRNA from specimens as soon as possible.

6.6.2. Resuspend RNA to a final concentration of 100 µg to 400 µg per mL in RNase-free molecular biology grade or USP water.

- This is a robust assay system; a wide range of RNA concentrations will generate a valid result.
- Therefore, quantifying and adjusting RNA concentrations is generally not necessary.

6.6.3. Synthesize cDNA from RNA, using the positive control RNA as a cDNA synthesis control (and use the cDNA from the positive control RNA as a positive PCR control when testing cDNA samples).

6.7. cDNA Synthesis Protocol

6.7.1. In a single microcentrifuge tube, mix the components following the table below:

Table 3. cDNA synthesis reaction set-up

Component	Volume per sample
RNA	24.0 µL
5X SuperScript first-strand buffer	10.0 µL
25 mM dNTPs	2.0 µL
water	3.0 µL
100 mM DTT	5.0 µL
random primers (3 µg/µL)	2.0 µL
Total volume	**46.0 µL**

6.7.2. Heat to 96°C to 100°C for 2 minutes and quick chill on ice.
6.7.3. Collect sample with a brief spin.
6.7.4. Add 2 µL of RNasin (5 units/µL) and mix.
6.7.5. Add 2 µL of SuperScript II and mix. (Final reaction volume of 50 µL.)
6.7.6. Place tubes at 42°C for 90 minutes.

Note: Use a heating block with a heated lid. If the heating block does not heat the caps, use a mineral overlay. Otherwise condensation will change the effective concentration in the solution.

6.7.7. Heat sample to 96°C to 100°C for 2 minutes and quick chill on ice. Collect sample at the bottom of the tube with a brief spin.

- Use cDNA as is, it is not necessary to dilute further.

6.7.8. Test the sample cDNA with the Specimen Control Size Ladder master mix to ensure that cDNA of sufficient quality and quantity was present to yield a valid result.

- The Specimen Control Size Ladder targets single gene exons (primers do not span intron/exon borders), and if RNA or cDNA is contaminated with gDNA, the Specimen Control Size Ladder may amplify the gDNA and produce results that are not indicative of the quality and quantity of the cDNA present.
- Remove gDNA contamination from RNA samples with DNase; however, properly inactivate or remove the DNase from the sample prior to cDNA synthesis.

6.8. RNA and cDNA Sample Storage

- Store RNA at -85°C to -65°C until use.
- Store cDNA at 2°C to 8°C or at -85°C to -65°C for long term storage.

7. Assay Procedure

7.1. Materials Provided

Table 4. Materials Provided

Gel Detection Kits	
Catalog #	**Description**
REF 21010170	Hypermutation Mix 1 v2.0 - Unlabeled
REF 21010180	Hypermutation Mix 2 v2.0 – Unlabeled
REF 20960020	Specimen Control Size Ladder – Unlabeled
REF 40880730	IVS-0013 Clonal Control DNA
REF 40891090	IVS-0013 Clonal Control RNA
REF 40920010	IVS-0000 Polyclonal Control DNA
REF 31010380	*IGH* J_H Primer – Unlabeled
REF 30000000	Primer – Hypermutation - Unlabeled

ABI Fluorescence Detection Kits	
Catalog #	**Description**
REF 21010171	Hypermutation Mix 1 v2.0 – 6FAM
REF 21010181	Hypermutation Mix 2 v2.0 – 6FAM
REF 20960021	Specimen Control Size Ladder – 6FAM
REF 40880730	IVS-0013 Clonal Control DNA
REF 40891090	IVS-0013 Clonal Control RNA
REF 40920010	IVS-0000 Polyclonal Control DNA
REF 31010380	*IGH* J_H Primer – Unlabeled
REF 30000000	Primer – Hypermutation - Unlabeled

7.2. Materials Required (not provided)

Table 5. Materials Required (not provided)

Reagent/Material	Recommended Reagents/Materials and Suppliers	Part Number	Notes
Reverse Transcriptase	Thermo Fisher Scientific®: • SuperScript II (SSII) RT	18064-071	Includes 100 mM DTT and 5X SuperScript first-strand buffer
25mM dNTP mix	Thermo Fisher Scientific: • Nucleotide Mix, 25mM	R1121	N/A
Random Primers	Thermo Fisher Scientific: • Random Primers	48190-011	N/A
Ribonuclease Inhibitor	Promega: • RNasin® Ribonuclease Inhibitor	N2115	N/A
DNA Polymerase	Roche: • EagleTaq DNA Polymerase Invivoscribe: • FalconTaq DNA Polymerase or equivalent	05206944190 60970130	N/A
Molecular Biology Grade or USP Water	N/A	N/A	DNase / RNase free
Calibrated Pipettes	N/A	N/A	Capable of accurately measuring volumes between 1 µL and 1000 µL.
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 Electrophoresis Unit	N/A	N/A	For agarose or polyacrylamide gels
Agarose	Thermo Fisher Scientific: • MetaPhor™ Agarose • NuSieve™ 3:1 Agarose	BMA50181 BMA50090	N/A

Table 5. Materials Required (not provided)

Reagent/Material	Recommended Reagents/Materials and Suppliers	Part Number	Notes
Ethidium Bromide	Thermo Fisher Scientific: • UltraPure® 10 mg/mL Ethidium Bromide	15595-011	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 Buffer (5X)	10816-015 LC6678	N/A
100 bp DNA Ladder	Thermo Fisher Scientific: • TrackIt® 100 bp DNA Ladder	10488-058	N/A
ABI Capillary Electrophoresis Instrument	Thermo Fisher Scientific: • ABI 310, 3100 or 3130 series	N/A	N/A
Hi-Di Formamide	Thermo Fisher Scientific: • Hi-Di® Formamide	4311320	N/A
ROX 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: • GeneScan™ - 400HD [ROX]™	60980051 60980061 402985	

Spectral Calibration Dye Set D	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]
		4345827
		
		402996
		
		401546
			Dye set used to spectrally calibrate ABI instrument for use with 6-FAM, 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
		402838
		4316355
		4352755
		
		4352759
			N/A

Buffer	Thermo Fisher Scientific: • 10X Genetic Analyzer Buffer with EDTA	402824	Dilute 1:10 in sterile water before use
Gel Extraction Kit	Qiagen: • MinElute Gel Extraction Kit • QIAEX II Gel Extraction Kit	28604 20021	N/A
TA Cloning Kit	Thermo Fisher Scientific: • TOPO TA Cloning® Kit (with pCR®2.1-TOPO® vector) with OneShot TOP10 Chemically Competent E. coli	K4500-01	N/A
Plasmid Isolation Kit	Qiagen: • QIAprep Spin MiniPrep Kit	27104	N/A

7.3. Reagent Preparation

- Test all unknown samples with the Specimen Control Size Ladder master mix to ensure that no inhibitors of amplification are present and there is gDNA or cDNA of sufficient quality and quantity to generate a valid result.
- Test positive, negative and no template controls with each master mix.

7.3.1. Using gloved hands, remove the master mixes from the freezer. Allow the tubes to thaw completely; then gently vortex to mix.

7.3.2. In containment hood or dead air box, remove an appropriate aliquot from each master mix to individual clean, sterile microcentrifuge tubes.

- Aliquot volumes are 45 µL for each reaction.
- Add an additional reaction volume for every 15 reactions to correct for pipetting errors. Thus, for each master mix (except for the Specimen Control Size Ladder), the number of reactions (**n**) is:

n =	**# of samples**	(run each sample in singlicate)
	+ 1	positive control DNA (required if testing gDNA samples)
	+ 1	cDNA from positive control RNA (required if testing cDNA samples)
	+ 1	negative control DNA (IVS-0000 Polyclonal Control DNA)
	+ 1	no template control (water)
	+ 1	to correct for pipetting errors
n =	**# of samples + 5**	**Total**

- Therefore, the total aliquot volume for each master mix is **n × 45 µL**.
- For the Specimen Control Size Ladder master mix, the number of reactions (**m**) can be calculated:

m =	**# of samples**	(run each sample in singlicate)
	+ 1	positive control DNA (IVS-0000 Polyclonal Control DNA)
	+ 1	no template control (water)
	+ 1	to correct for pipetting errors
m =	**# of samples + 3**	**Total**

- Therefore the total aliquot volume for the Specimen Control Size Ladder master mix is **m × 45 µL**.

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

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

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

7.3.5. Add 5 µL of appropriate template (sample gDNA or cDNA, positive control DNA or cDNA, negative control DNA or water) to the individual wells containing the respective master mix solutions. Pipette up and down several times to mix.

7.3.6. Cap or cover the PCR plate.

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

7.3.7. If amplification cannot be performed immediately following reagent preparation, the PCR plate or tubes can be stored at 2ºC to 8ºC for up to 24 hours.

Quick Guide

For each master mix and n reactions, mix:

n × 45 µL	Master Mix
n × 0.25 µL	Taq DNA polymerase

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

7.4. Amplification

7.4.1. Amplify the samples using the following PCR program:

- Use the **calculated** option for temperature measurement with the BioRad MJ Research PTC thermal cyclers.

Table 6. Thermal cycling conditions

Step	Temperature	Duration	Cycles
1	95°C	7 minutes	1
2	95°C	45 seconds	35
3	60°C	45 seconds	
4	72°C	90 seconds	
5	72°C	10 minutes	1
6	15°C	∞	1

7.4.2. Remove the amplification plate or tubes from the thermal cycler.

7.4.3. Proceed with one of the following detection methods in section 7.5 *Detection*.

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

7.5. Detection

Agarose Gel Detection

- This section is for detection of samples amplified with Unlabeled Master Mixes.

7.5.1. Prepare a 2% MetaPhor or NuSieve 3:1 agarose/1X TBE gel with large combs.

7.5.2. Place gel in electrophoresis unit and cover with 1X TBE buffer.

7.5.3. Mix 20 µL of each PCR product with 4 µL of 6X gel loading buffer.

7.5.4. Load 20 µL of this mixture into separate wells of the gel and 4 µL of the 100 bp DNA Ladder flanking the samples.

7.5.5. Run at 100V for 1.5 to 2 hours.

- Voltage and electrophoresis time depend on the PCR amplicon size, gel length and % of agarose in the gel.
- Voltage and run time can be adapted accordingly.

7.5.6. Stain gel with ethidium bromide or other dye.

7.5.7. Place gel over UV illuminator and photograph data.

7.5.8. Interpret data. (See section 8: *Data Interpretation*)

7.5.9. Proceed with sequencing. (See section 7.8 *Sequencing of PCR Product*)

Polyacrylamide Gel Detection

- This section is for detection of samples amplified with Unlabeled Master Mixes.

7.5.10. Assemble the electrophoresis unit using a non-denaturing, 6% polyacrylamide TBE gel and 1X TBE running buffer.
7.5.11. Mix 20 µL of each sample with 5 µL of ice-cold non-denaturing 5X bromophenol blue loading buffer.
7.5.12. Load all 20 µL of the mixture into individual wells of the gel.
7.5.13. Run gel at 110 V for 2-3 hours or 40-50V overnight.

- Voltage and electrophoresis time depend on the PCR amplicon size and polyacrylamide gel thickness.
- Voltage and run time can be adapted accordingly.

7.5.14. Stain gels in 0.5 µg/mL ethidium bromide (in water or 0.5X TBE Buffer) for 5-10 minutes.
7.5.15. De-stain gels in water for 5-10 minutes. Repeat with new water.
7.5.16. Place gel over UV illuminator and photograph data.
7.5.17. Interpret data. (See section 8: *Data Interpretation*)
7.5.18. Proceed with sequencing. (See section 7.8: *Sequencing of PCR Product*)

Heteroduplex Analysis

- This section is for detection of samples amplified with Unlabeled Master Mixes.

7.5.19. Denature 20 µL of PCR products at 94ºC for 5 minutes.
7.5.20. Quick chill PCR products at 4ºC (on an ice water bath) for 60 minutes.
7.5.21. Proceed with polyacrylamide gel detection, in step 7.5.10.

ABI Fluorescence Detection

Note: When using ABI instruments for fluorescence detection, there can often be a preceding peak, which is an artifact due to the detection method the ABI platforms use. Preceding peaks are sometimes skewed and have bases that slope on the right side towards the real peak. This is especially evident in the Specimen Control Size Ladder master mix where the 96 nt peak has a preceding peak that occurs at 84 nt.

7.5.22. In a new microcentrifuge tube, mix an appropriate amount (for a total of 10 µL per reaction) of Hi-Di Formamide with ROX Size Standards. Vortex well.
7.5.23. In a new 96-well PCR plate, add 10 µL of Hi-Di Formamide with ROX size standards to individual wells for each reaction.
7.5.24. 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.
7.5.25. Cap or cover the PCR plate or tubes.
7.5.26. Heat denature the samples at 95ºC for 2 minutes then snap chill on ice for 5 minutes.
7.5.27. Prepare a **sample sheet** and **injection list** for the samples.
7.5.28. Run the samples on an ABI capillary electrophoresis instrument according to the user manual.

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

7.5.29. Review profile and controls, report results. (see section 8: *Data Interpretation*)
7.5.30. Proceed with sequencing. (see section 7.8: *Sequencing of PCR Product*)

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. In addition, include a no template control (*e.g.* water) to test for contamination of the master mix or cross-contamination of reactions due to improper sterile technique. A buffer control may also be added to ensure that no contamination of the buffer used to resuspend the samples has occurred. The values for the positive controls are provided in Table 7. Additional controls and sensitivity controls (dilutions of positive controls into negative control) are available from Invivoscribe.

7.7. Recommended Positive Controls

The amplicon sizes listed were determined using an ABI instrument. Amplicon sizes seen on each specific capillary electrophoresis instrument may differ 1 to 4 nucleotides (nt) from those listed depending on the platform of detection and the version of the analysis software used. Once identified, the amplicon size as determined on each specific platform will be consistent from run to run. This reproducibility is extremely useful when monitoring disease recurrence.

Note: "Color" indicates the color of products generated with the master mix when using the default color assignment on ABI fluorescence detection systems.

Table 7. Recommended Positive Controls

Master Mix	Target	Color	Control RNA and DNA	DNA Catalog #	DNA Product Size (nt)	RNA Catalog #	RNA Product Size (nt)
Hypermutation Mix 1 v2.0	Leader to J_H	**Blue**	**Valid Size Range** IVS-0013 Clonal Control	--- 40880730	**500 - 570** 536	--- 40891090	**415 – 485** 461
Hypermutation Mix 2 v2.0	FR1 to J_H	**Blue**	**Valid Size Range** IVS-0013 Clonal Control	--- 40880730	**310 - 380** 358	--- 40891090	**310 - 380** 358
Specimen Control Size Ladder	Multiple Genes	**Blue**	**Valid Size Range** IVS-0000 Polyclonal Control	--- 40920010	**96 - 602** 96, 197, 297, 397, 602	n/a	**same as DNA**

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

7.8. Sequencing of PCR Product

7.8.1. Direct Sequencing

Direct sequencing works best for samples with little to no background amplification and only one clonal product.

7.8.1.1. In two separate containers, prepare the appropriate concentration of each sequencing primer with the PCR product:

- *IGH* J_H Primer – Unlabeled and
- Primer – Hypermutation – Unlabeled

7.8.1.2. Sequence the sample.

7.8.1.3. Proceed with section 11: *Hypermutation Analysis and Reporting*.

7.8.2. Gel Extraction

Use gel extraction with weak clonal bands in a polyclonal background or if there is more than one clonal band.

7.8.2.1. After gel data has been analyzed and clonal bands identified, place gel over UV illuminator.

Note: UV light is harmful and damaging to eyes and skin. Please use caution and wear a UV shield, lab coat and gloves to cover eyes and exposed skin.

7.8.2.2. Use a clean razor blade, scalpel or band pick to remove positive band from gel, avoid removing excess gel and be careful not to cut into other bands as they will contaminate the sample.

7.8.2.3. Use a gel extraction kit to extract and elute the DNA in an appropriate buffer at a high concentration.

7.8.2.4. Proceed with either Direct Sequencing, above, or Cloning and Sequencing below.

7.8.3. Cloning and Sequencing

Clone the band extracted PCR product using Invitrogen's TOPO TA Cloning Kit. Please note that cloning is only reliable when performed with unlabeled amplicons.

7.8.3.1. Use 4 µL of the band extracted PCR product for the ligation reaction with the pCR2.1-TOPO vector.
7.8.3.2. Follow the manufacturer's protocol for transforming and plating bacteria.
7.8.3.3. Pick at least 8 white colonies per band and grow in 2-4 mL of LB + ampicillin media in a shaking incubator at 37°C overnight.
7.8.3.4. Spin each bacterial culture down at 6000 rpm in a centrifuge and pour off media.
7.8.3.5. Proceed with a plasmid DNA extraction using the QIAgen QIAprep Spin Miniprep Kit.
7.8.3.6. Sequence each plasmid sample with M13 forward and/or M13 reverse sequencing primers.
7.8.3.7. Proceed with section 11 *Hypermutation Analysis and Reporting*.

8. Data Interpretation

This assay is for research use only; not intended for diagnostic purposes. PCR based testing does not identify 100% of clonal cell populations; therefore, repeat testing by NGS may be advisable to rule out clonality. For accurate and meaningful interpretation ignore peaks that occur outside of the valid size range for each master mix.

8.1. Analysis

8.1.1. Samples that fail to amplify following repeat testing can be reported as "A result cannot be reported on this specimen because there was DNA of insufficient quantity or quality for analysis".
8.1.2. If samples run in duplicate yield differing results, re-test and/or re-evaluate samples for sample switching.
8.1.3. Examine all assay controls prior to interpretation of sample results.
8.1.4. The following table describes the analysis of each control and the decisions based upon the results.

Table 8. Control Analysis Summary

Type of Control	Expected Result	Aberrant Result
No Template Control	No amplification present, continue with analysis	Amplification present. Repeat the assay.
Polyclonal Control	Product size is consistent with expected size listed in Table 9. No clonal rearrangements are present. Continue with analysis.	Clonal rearrangements are present. Repeat the assay.
Positive Control (This can also be an extraction control if positive control material is taken through extraction processes)	Product size is consistent with expected size listed in Table 9. Continue with analysis.	Repeat the assay.
Specimen Control Size Ladder (This amplification control is essential for samples of unknown quantity and quality.)	If all of the 96, 197, 297, 397, and 602 nt peaks are observed, continue with analysis. Because smaller PCR fragments are preferentially amplified, it is not unusual for the 608 nt product to have a diminished signal or to be missing entirely. Continue with analysis.	If no bands are seen, repeat the assay. If only 1, 2, or 3 bands are seen, re-evaluate sample for DNA degradation unless specimen tests positive.

8.2. Sample Interpretation

Given that the controls produce expected results, interpret the samples as follows:

8.2.1. One or two prominent positive peaks[a] within the valid size range indicates the sample is "Positive" for the "detection of clonal immunoglobulin heavy chain gene rearrangement(s), consistent with the presence of a clonal cell population."

8.2.2. An absence of positive peaks[a] within the valid size range indicates the sample is "**Negative**" for the "**detection of clonal immunoglobulin heavy chain gene rearrangement(s)**."

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

- **ABI Fluorescence Detection:** Products generated from samples that fall within the valid size range and are at least three times the amplitude of the third largest peak in the polyclonal background are consistent with a positive peak.
- **Gel Detection:** Products generated from samples that fall within the valid size range and produce a discrete band(s) distinct from any background smear are consistent with a positive peak.

8.2.3. Extract the positive samples from the gel and sequence (if ABI Fluorescence detection was initially used, amplify with the unlabeled master mixes, available for purchase from Invivoscribe for gel detection and run on a gel.; see section 7.8: *Sequencing of PCR Product*).

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 Results

10.1. Expected Size of Amplified Products

The amplicon sizes listed were determined using an ABI platform. Amplicon sizes observed on each specific capillary electrophoresis instrument may differ 1 to 4 nucleotides (nt) from those listed depending on the platform of detection and the version of the analysis software used. Once identified, the amplicon size as determined on 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 9. Expected Size of Amplified Products

Master Mix	Target	Color	Control DNA or RNA	Catalog #	Product Size (nt)
Hypermutation Mix 1 v2.0	Leader to J_H	**Blue**	**Valid Size Range (DNA)** IVS-0000 Polyclonal Control DNA IVS-0013 Clonal Control DNA **Valid Size Range (RNA)** IVS-0013 Clonal Control RNA	--- 40920010 40880730 40891090	**500 - 570** 500 - 570 536 **415 - 485** 461
Hypermutation Mix 2 v2.0	FR1 to J_H	**Blue**	**Valid Size Range (DNA)** IVS-0000 Polyclonal Control DNA IVS-0013 Clonal Control DNA **Valid Size Range (RNA)** IVS-0013 Clonal Control RNA	--- 40920010 40880730 40891090	**310 - 380** 310 - 380 358 **310 - 380** 358
Specimen Control Size Ladder	Multiple Genes	**Blue**	**Valid Size Range (DNA & RNA)** Any Human DNA or cDNA	---	**96 – 602** 96, 197, 297, 397, 602

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

10.2. Sample Data

Gel Detection: The data shown in Figure 2 and Figure 3 were generated using the indicated master mixes and run on a gel.

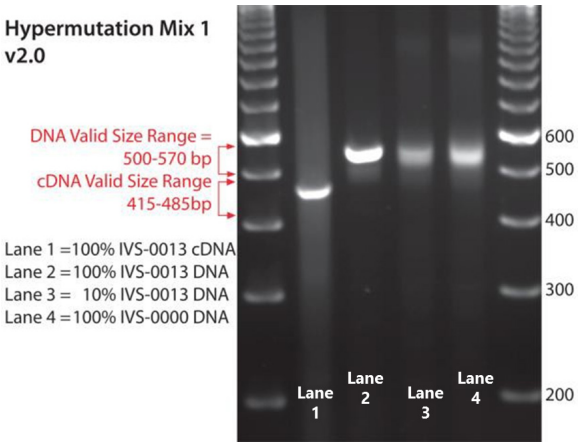

Figure 2. Amplified products were generated using Hypermutation Mix 1 v2.0

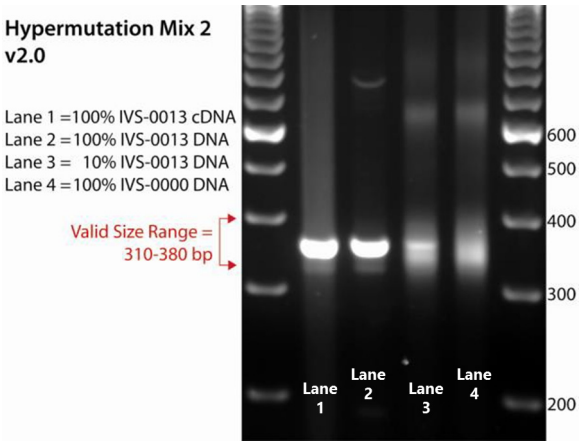

Figure 3. Amplified products were generated using Hypermutation Mix 2 v2.0.

ABI Fluorescence Detection: The data shown in Figure 4 - Figure 6 were generated using the master mixes indicated and run on an ABI instrument.

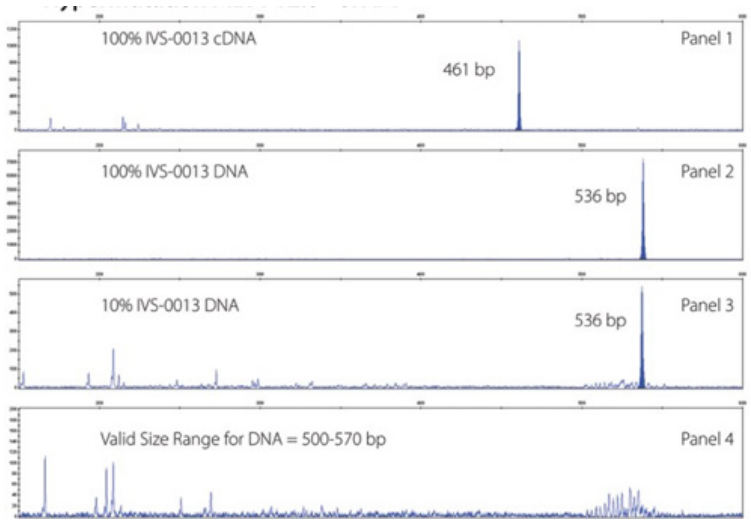

Figure 4. Amplified products were generated using Hypermutation Mix 1 v2.0 – 6FAM.

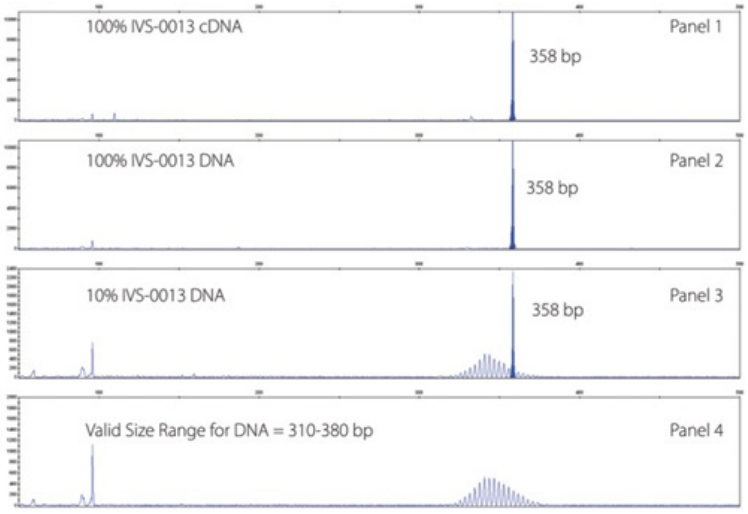

Figure 5. Amplified products were generated using Hypermutation Mix 2 v2.0 – 6FAM.

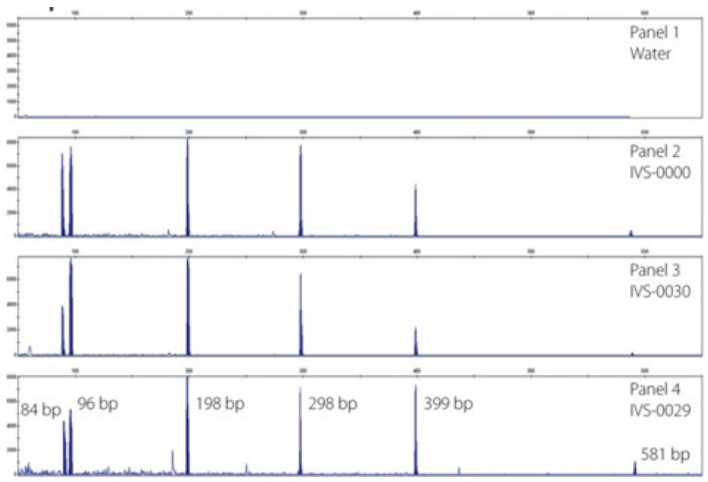

Figure 6. Products were generated using the Specimen Control Size Ladder master mix.

11. Hypermutation Analysis and Reporting

11.1. Analysis

11.1.1. Align multiple sequence data obtained from a given sample and check for concordance. The majority of the sequences will be identical.

- For sequences obtained by cloning, ignore vector sequences.
- For sequences obtained by direct sequencing, adjust sequences so that they are in the V to J orientation instead of J to V.

11.1.2. Check sequences to ensure they represent real V-D-J product(s). This can be done with the aid of one or more of the websites listed below:

- V BASE – The MRC Centre for Protein Engineering's Database of human antibody genes
  - http://www.vbase2.org
  - Analysis tools: DNAPLOT
- NCBI – National Center for Biotechnology Information
  - http://www.ncbi.nlm.nih.gov/igblast/
  - Analysis tools: IgBLAST (Basic Local Alignment Search Tool)
- Please note different databases may produce varying results and may have different amino acid numbering definitions for FR and CDR regions.

11.1.3. After identifying a valid sequence, *IGH* somatic hypermutation analysis can be performed using one of the listed websites; alternatively, this can be performed manually.

- If the sequence was obtained using the Hypermutation Mix 1 v2.0, analysis of the full FR1-FR3 region can be completed.
- If the sequence was obtained using the Hypermutation Mix 2 v2.0, analysis of only the CDR1-FR3 region can be completed.

11.1.4. Find the germline V region sequence that best corresponds to the sample sequence.

11.1.5. Align the germline V region sequence to the V region sequence of the sample.

11.1.6. Determine the number of mismatched bases and the total number of bases that are being compared.

- $\%\, divergence = \frac{number\ of\ mismatched\ bases}{total\ number\ of\ bases\ compared}$
- $\%\, homology = 100\% - \%\, divergence$

11.1.7. Complete these steps for both the forward and reverse sequences.

11.1.8. If the forward and reverse sequences are discordant for mutational status, repeat the experiment.

11.2. IVS-0013 Clonal Control Data

Sequence data obtained from the positive IVS-0013 Clonal Control DNA or RNA will correspond to an unmutated, in-frame V_H1-46 to J_H4 rearrangement.

11.3. Sample Data

The following sequence data is an example of an alignment using V BASE's DNAPLOT analysis tool and the sequence data of a sample. The sample was sequenced using the Hypermutation Mix 2, therefore only data from CDR1 to FR3 (amino acids 31 to 95) was used. DNAPLOT gives five germline sequences with the highest degree of homology. In this case, the sequence has the highest degree of homology with the germline sequence from DP-75 which corresponds to V_H1-02.

- $\textit{The \% divergence for this sample} = \frac{21}{206} = 10.2\%$

EMBL	Locus	Name	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Z14071	1-02	DP-75/VI-2...+	CAG GTG CAG CTG GTG CAG TCT GGG GCT GAG GTG AAG AAG CCT GGG GCC TCA GTG AAG GTC TCC
X92208	1-02	1-1+	CAG GTG CAG CTG GTG CAG TCT GGG GCT GAG GTG AAG AAG CTT GGG GCC TCA GTG AAG GTC TCC
Z12310	1-02	DP-8+	CAG GTG CAG CTG GTG CAG TCT GGG GCT GAG GTG AAG AAG CCT GGG GCC TCA GTG AAG GTC TCC
Z14213	-	V_HG_L1.2	CAG GTG CAG CTG GTG CAG TCT GGG GCT GAG GTG AAG AAG CCT GGG GCC TCA GTG AAG GTT TCC
X07448	1-02	V35/VI-2b+	CAG GTG CAG CTG GTG CAG TCT GGG GCT GAG GTG AAG AAG CCT GGG GCC TCA GTG AAG GTC TCC

			H1 CDR1
EMBL	Locus	Name	22 23 24 25 26 27 28 29 30 31 31a 31b 32 33 34 35 36 37 38 39 40
			... ... GAC ... ... TAC TAT TTG CAC TGG GTG CGA CAG GCC
Z14071	1-02	DP-75/VI-2...+	TGC AAG GCT TCT GGA TAC ACC TTC ACC -G- ... ... --- --- A-- --- --- --- --- --- ---
X92208	1-02	1-1+	TGC AAG GCT TCT GGA TAC ACC TTC ACC -G- ... ... --- --- A ---------- X- ----
Z12310	1-02	DP-8+	TGC AAG GCT TCT GGA TAC ACC TTC ACC -G- ... ... --- --- A-- --- --- --- --- --- ---
Z14213	-	V_HG_L1.2	TGC AAG GCA TCT GGA TAC ACC TTC ACC AG- ... ... --- --- A-- --- --- --- --- --- ---
X07448	1-02	V35/VI-2b+	TGC AAG GCT TCT GGA TAC ACC TTC ACC -G- ... ... --- --- A-- --- --- --- --- --- ---

			H2 CDR2
EMBL	Locus	Name	41 42 43 44 45 46 47 48 49 50 51 52 52a 52b 52c 53 54 55 56 57 58
			CCT GGA CAA GGG TTT GAG TGG ATG GGA TGG ATC AAC CTT ... ... AAG AAT GGT GCC ATC AGG
Z14071	1-02	DP-75/VI-2...+	--- --- --- --- C-- --- --- --- --- --- --- --- -C- ... ... --C -G- --- -G- -CA -AC
X92208	1-02	1-1+	--- --- --- --- C-- --- --- --- --- --- --- --- -C- ... ... --C -G- --- -G- -CA -AC
Z12310	1-02	DP-8+	--- --- --- --- C-- --- --- --- --- --- --- --- -C- ... ... --C -G- --- -G- -CA -AC
Z14213	-	V_HG_L1.2	--- --- --- --- C-- --- --- --- --- --- --- --- -C- ... ... --C -G- --- -G- -CA -AC
X07448	1-02	V35/VI-2b+	--- --- --- --- C-- --- --- --- --- C-- --- --- -C- ... ... --C -G- --- -G- -CA -AC

EMBL	Locus	Name	59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79
			TAT GCA CAG AGG TTT GAG GAC AGG GTC ACC ATG ACC AGT GAC ACG TCC ATT AGC ACA GCC TAC
Z14071	1-02	DP-75/VI-2...+	--- --- --- -A- --- C-- -G- --- --- --- --- --- --G --- --- --- --C --- --- --- ---
X92208	1-02	1-1+	--- --- --- -A- --- C-- -G- --- --- --- --- --- --G --- --- --- --C --- --- --- ---
Z12310	1-02	DP-8+	--- --- --- -A- --- C-- -G- T-- --- --- --- --- --G --- --- --- --C --- --- --- ---
Z14213	-	V_HG_L1.2	--- --- --- -A- --- C-- -G- --- --- --- --- --- --G --- --- --- --C --- --- --- ---
X07448	1-02	V35/VI-2b+	--- --- --- -A- --- C-- -G- --- --- --- -GT --- --G --- --- --- --C --- --- --- ---

EMBL	Locus	Name	80 81 82 82a 82b 82c 83 84 85 86 87 88 89 90 91 92 93 94 95
			ATG GAG CTG GGC AGG CTG AAA TCA GAC GAC ACG GCT GTA TAT TAC TGT GCG AGA GAG
Z14071	1-02	DP-75/VI-2...+	--- --- --- A-- --- --- -G- --T --- --- --- --C --G --- --- --- --- --- ---
X92208	1-02	1-1+	--- --- --- A-- --- --- -G- --T --- --- --- --C --G --- --- --- --- --- ---
Z12310	1-02	DP-8+	--- --- --- A-- --- --- -G- --T --- --- --- --C --G --- --- --- --- --- ---
Z14213	-	V_HG_L1.2	G-- --- --- A-- --- --- -G- --T --- --- --- --C --G --- --- --- --- --- ---
X07448	1-02	V35/VI-2b+	--- --- --- A-- --- --- -G- --T --- --- --- -TC --G --- --- --- --- --- ---

The following is the same sequence analyzed with the NCBI IgBlast tool. Again only data from CDR1 to FR3 (amino acids 31 to 95) was used, and the sequence was also found to have the highest degree of homology with V_H1-02 (additional sequences were listed, but only the top three matches are shown below). IgBlast provides the percentage of identity (or homology) given by (ID%). This sample is 90% homologous to the germline V_H1-02.

- $\%\ divergence = 100\% - 90\% = 10\%$

```
ID%   tmpseq_0    3     <-----CDR1---->  <-------------------FWR2----------------->  <----------------------
                        --CTACTATTTGCAC  TGGGTGCGACAGGCCCCTGGACAAGGGTTTGAGTGGATGGGA  TGGATCA-ACCTTAAGAATGGTG 79
90    VH1-2       91    GG.......A.....  ............................C..............  .......-...C...C.G......169
86    VH1-8       104   -------------..  ...............A...........C..............  .....G.-...C...C.G....A 169
84    VH1-46      93    --.......A.....  ............................C..............  ATA....-...C..GTGG....A 169

ID%   tmpseq_0    80    --CDR2-----------------------> <-----------------------------------------FWR
                        CCATCAGG-TATGCACAGAGGTTTGAGGAC  AGGGTCACCATGACCAGTGACACGTCCATTAGCACAGCCTACATGGAGCT 158
90    VH1-2       170   G..CA.AC-..........A....C...G.  .................G...........C.. 248
86    VH1-8       170   A..-....C..........A...CC...G.  ..A..............GA....C.....A.. 248
84    VH1-46      170   G..CA..C-..C.......A...CC...G.  ..A..............G..........CG.......T.      248

ID%   tmpseq_0    159   3------------------------------------------>
                        GGGCAGGCTGAAATCAGACGACACGGCTGTATATTACTGTGCGAGA204
90    VH1-2       249   .A.........G...T...........C..G.             294
86    VH1-8       249   .A....C....G...T..G........C..G.             294
84    VH1-46      249   .A....C....G...T..G........C..G.             294
```

The following is the same sequence analyzed with IMGT's V-QUEST tool. Again only data from CDR1 to FR3 was used, however, IMGT has a different delineation for FR and CDR regions. Therefore the CDR1 - FR3 region used for this analysis corresponded to amino acids 27 through 104. Again the sequence was found to have the highest degree of homology with V_H1-02 (additional sequences are listed).

- $\%\ divergence = \frac{25}{231} = 10.8\%$

```
                        <----------------------  F   R   1   -   I   M   G   T  ---------------
                        1   2   3   4   5   6   7   8   9   10  11  12  13  14  15  16  17  18
input                   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
X62106 IGHV1-2*02       CAG GTG CAG CTG GTG CAG TCT GGG GCT ... GAG GTG AAG AAG CCT GGG GCC TCA
X92208 IGHV1-2*03       CAG GTG CAG CTG GTG CAG TCT GGG GCT ... GAG GTG AAG AAG CTT GGG GCC TCA
Z12310 IGHV1-2*04       CAG GTG CAG CTG GTG CAG TCT GGG GCT ... GAG GTG AAG AAG CCT GGG GCC TCA
X07448 IGHV1-2*01       CAG GTG CAG CTG GTG CAG TCT GGG GCT ... GAG GTG AAG AAG CCT GGG GCC TCA
L06612 IGHV1-46*03      CAG GTG CAG CTG GTG CAG TCT GGG GCT ... GAG GTG AAG AAG CCT GGG GCC TCA

                        ------------------------------->
                                                          _____________________CDR1 - IMGT________
                        19  20  21  22  23  24  25  26  27  28  29  30  31  32  33  34  35  36
input                   ... ... ... ... ... ... ... ... AGA AAC ACA TTC AGC GAC TAC TAT ... ...
X62106 IGHV1-2*02       GTG AAG GTC TCC TGC AAG GCT TCT G-- T-- --C --- -C- -G- --- --- ... ...
X92208 IGHV1-2*03       GTG AAG GTC TCC TGC AAG GCT TCT G-- T-- --C --- -C- -G- --- --- ... ...
Z12310 IGHV1-2*04       GTG AAG GTC TCC TGC AAG GCT TCT G-- T-- --C --- -C- -G- --- --- ... ...
X07448 IGHV1-2*01       GTG AAG GTC TCC TGC AAG GCT TCT G-- T-- --C --- -C- -G- --- --- ... ...
L06612 IGHV1-46*03      GTG AAG GTT TCC TGC AAG GCA TCT G-- T-- --C --- -C- AG- --- --- ... ...

                                  <------------------  F   R   2   -   I   M   G   T   --------
                                  _________
                        37  38  39  40  41  42  43  44  45  46  47  48  49  50  51  52  53  54
input                   ... ... TTG CAC TGG GTG CGA CAG GCC CCT GGA CAA GGG TTT GAG TGG ATG GGA
X62106 IGHV1-2*02       ... ... A-- --- --- --- --- --- --- --- --- --- --- C-- --- --- --- ---
X92208 IGHV1-2*03       ... ... A-- --- --- --- -X- --- --- --- --- --- --- C-- --- --- --- ---
Z12310 IGHV1-2*04       ... ... A-- --- --- --- --- --- --- --- --- --- --- C-- --- --- --- ---
X07448 IGHV1-2*01       ... ... A-- --- --- --- --- --- --- --- --- --- --- C-- --- --- --- ---
L06612 IGHV1-46*03      ... ... A-- --- --- --- --- --- --- --- --- --- --- C-- --- --- --- ---

                        -->                                       <----------------------------
                                  ___________CDR2 - IMGT _________________
                        55  56  57  58  59  60  61  62  63  64  65  66  67  68  69  70  71  72
input                   TGG ATC AAC CTT AAG AAT GGT GCC ATC ... ... AGG TAT GCA CAG AGG TTT GAG
X62106 IGHV1-2*02       --- --- --- -C- --C -G- --- -G- -CA ... ... -AC --- --- --- -A- --- C--
X92208 IGHV1-2*03       --- --- --- -C- --C -G- --- -G- -CA ... ... -AC --- --- --- -A- --- C--
Z12310 IGHV1-2*04       --- --- --- -C- --C -G- --- -G- -CA ... ... -AC --- --- --- -A- --- C--
X07448 IGHV1-2*01       C-- --- --- -C- --C -G- --- -G- -CA ... ... -AC --- --- --- -A- --- C--
L06612 IGHV1-46*03      ATA --- --- -C- -GT GG- --- AG- -CA ... ... --C --C --- --- -A- --C C—
```

```
                        ------------------------  F   R   3   -   I   M   G   T  ------------
                        73  74  75  76  77  78   79  80  81  82  83  84  85  86  87  88  89

input                   ... GAC AGG GTC ACC ATG ACC AGT GAC ACG TCC ATT AGC ACA GCC TAC ATG GAG
X62106 IGHV1-2*02       ... -G- --- --- --- --- --- --G --- --- --- --C --- --- --- --- --- ---
X92208 IGHV1-2*03       ... -G- --- --- --- --- --- --G --- --- --- --C --- --- --- --- --- ---
Z12310 IGHV1-2*04       ... -G- T-- --- --- --- --- --G --- --- --- --C --- --- --- --- --- ---
X07448 IGHV1-2*01       ... -G- --- --- --- -GT --- --G --- --- --- --C --- --- --- --- --- ---
L06612 IGHV1-46*03      ... -G- --A --- --- --- --- --G --- --- --- -CG --- --- -T- --- --- ---
```

```
                        ------------------------------------------------------>
                                                                              ______
                        90  91  92  93  94  95  96  97  98  99  100 101 102 103 104 105
input                   CTG GGC AGG CTG AAA TCA GAC GAC ACG GCT GTA TAT TAC TGT GCG AGA
X62106 IGHV1-2*02       --- A-- --- --- -G- --T --- --- --- --C --G --- --- --- --- ---
X92208 IGHV1-2*03       --- A-- --- --- -G- --T --- --- --- --C --G --- --- --- --- ---
Z12310 IGHV1-2*04       --- A-- --- --- -G- --T --- --- --- --C --G --- --- --- --- ---
X07448 IGHV1-2*01       --- A-- --- --- -G- --T --- --- --- -TC --G --- --- --- --- ---
L06612 IGHV1-46*03      --- A-- --C --- -G- --T --G --- --- --C --G --- --- --- --T ---
```

Data from VBase2's DNAPLOT (not shown) is similar to V Base, however, Vbase2 uses the IMGT delineations for FR and CDR regions.

12. Technical and Customer Service

Thank you for purchasing our ***IGH* Somatic Hypermutation Assay v2.0**. 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. Questions received during business hours usually receive a response within an hour.

Contact Information

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Technical Service: support@invivoscribe.com | Customer Service: sales@invivoscribe.com | Website: www.invivoscribe.com

13. References

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14. Symbols

The following symbols are now used in labeling for Invivoscribe NGS diagnostic products.

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

15. Legal Notice

15.1. Warranty and Liability

Invivoscribe, Inc. (Invivoscribe®) is committed to providing the highest quality products. Invivoscribe® warrants that the products meet or exceed the performance standards described in the Instructions For Use, as to products with such an insert. If a product is covered by product specifications and does not perform as specified, our policy is to replace the product or credit the full purchase price. No other warranties of any kind, expressed or implied, are provided by Invivoscribe®. Invivoscribe® liability shall not exceed the purchase price of the product. Invivoscribe shall have no liability for direct, indirect, consequential or incidental damages arising from the use, results of use, or inability to use its products; product efficacy under purchaser controlled conditions in purchaser's laboratory must be established and continually monitored through purchaser defined and controlled processes including but not limited to testing of positive, negative, and blank controls every time a sample is tested. Ordering, acceptance, and use of product constitutes purchaser acceptance of sole responsibility for assuring product efficacy and purchaser agreement to the limitation of liability set forth in this paragraph.

This product is for Research Use Only; not for use in diagnostic procedures.

15.2. Patents and Trademarks

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