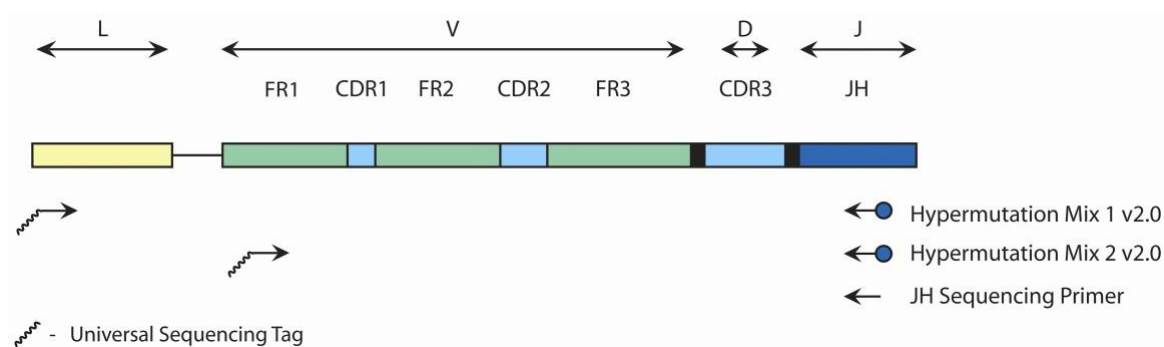


Instructions for Use

IGH Somatic Hypermutation Assay v2.0

To sequence and identify mutational status of clonal *IGH* gene rearrangements.

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



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Manufactured in U.S.A.



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

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

Catalog #	Products	Quantity
REF 5-101-0030	<i>IGH</i> Somatic Hypermutation Assay v2.0 for Gel Detection	33 Reactions
REF 5-101-0031	<i>IGH</i> Somatic Hypermutation Assay v2.0 for ABI Fluorescence Detection	33 Reactions
REF 5-101-0040	<i>IGH</i> Somatic Hypermutation Assay MegaKit v2.0 for Gel Detection	330 Reactions
REF 5-101-0041	<i>IGH</i> Somatic Hypermutation Assay MegaKit v2.0 for ABI Fluorescence Detection	330 Reactions

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1. Proprietary Name

IGH Somatic Hypermutation Assay v2.0 - Gel Detection

IGH Somatic Hypermutation Assay MegaKit v2.0 - Gel Detection

IGH Somatic Hypermutation Assay v2.0 - ABI Fluorescence Detection

IGH Somatic Hypermutation Assay MegaKit v2.0 - ABI Fluorescence Detection

2. Intended Use

The *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 in patients with chronic lymphocytic leukemia (CLL) and small lymphocytic lymphoma (SLL).

Specifically, the *IGH* Somatic Hypermutation Assay v2.0 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

3. Summary and Explanation of the Test

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 loci¹. 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 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 JH reverse primer. Current ERIC (European Research Initiative on CLL) guidelines are to employ bi-directional sequencing when determining the *IGH* somatic hypermutation (SHM) status⁴.

Immunoglobulin variable heavy chain gene hypermutation status provides important prognostic information for patients with chronic lymphocytic leukemia (CLL) and small lymphocytic lymphoma (SLL). 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. This has clinical relevance, as there is a clear distinction in the median survival of patients with and without somatic hypermutation. Hypermutation of the *IGHV* gene is strongly predictive of a good prognosis while lack of mutation predicts a poor prognosis. It should be emphasized that the results of any molecular test should always be interpreted in the context of clinical, histological and immunophenotypic data.

4. Principles of the Procedure

4.1. 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 genomic DNA 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 genomic DNA 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.

This test amplifies either genomic DNA 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 employs two 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.

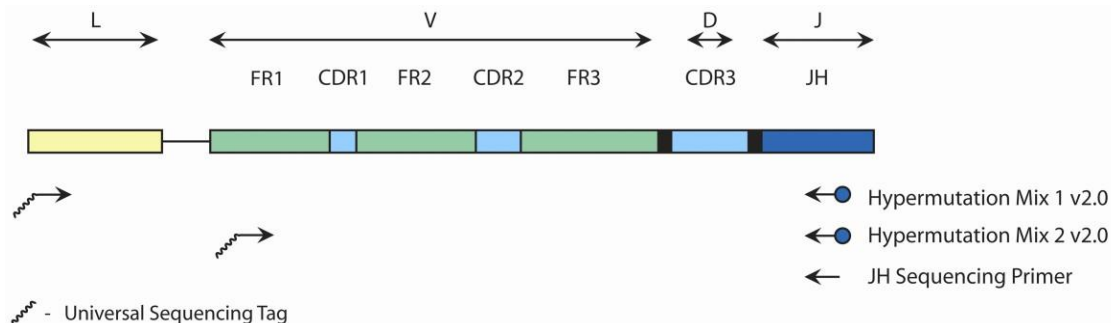


Figure 1. Depicted is a simple representation of the organization of a rearranged immunoglobulin heavy chain gene on chromosome 14. Black arrows represent the relative positions of primers that target the conserved Leader (L) and Framework 1 (FR1) regions and the downstream consensus JH gene segments.

4.2. Gel Electrophoresis Detection

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

4.3. 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 (fluorophors) so that they can produce different emission spectra upon excitation by a laser in the capillary electrophoresis instrument. In this manner, different fluorescent dyes can correspond to different targeted regions. This detection system results in unsurpassed sensitivity, single nucleotide resolution, differential product detection and relative quantification. In addition, the use of agarose and polyacrylamide gels, as well as the use of carcinogens such as ethidium bromide, can virtually be eliminated. Further, differential detection allows accurate, reproducible and objective interpretation of primer-specific products and automatic archiving of data. Inter-assay and intra-assay reproducibility in size determination using capillary electrophoresis is approximately 1 to 2 nucleotides. This reproducibility and sensitivity coupled with the automatic archiving of specimen data allows for the monitoring, tracking and comparison of data from individual patients over time.

4.4. *IGH* Somatic Hypermutation (SHM) Analysis

The degree of somatic mutation in the immunoglobulin heavy (*IGH*) chain variable (V) genes is one of the best prognostic tools in the treatment of patients with Chronic Lymphocytic Leukemia (CLL) and Small Lymphocytic Lymphoma (SLL). Clonal PCR products are identified and they are gel extracted and sequenced. For somatic hypermutation (SHM) analysis the full variable region (FR1-FR3) or a partial variable region (CDR1-FR3) are sequenced to determine mutational status. Mutational status is determined by comparing the sequence of the *IGH* V region of the patient 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. Listed below are several websites available to aid in *IGH* Somatic Hypermutation Analyses:

- IMGT – The International ImMunoGeneTics information system (Initiator and coordinator: Marie-Paule Lefranc, Montpellier, France) <http://imgt.cines.fr>
Analysis tools: IMGT/V-QUEST and IMGT/Junction Analysis
- V BASE – The MRC Centre for Protein Engineering’s Database of human antibody genes <http://vbase.mrc-cpe.cam.ac.uk/> and <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) For the latest recommendations for CLL hypermutation testing visit:
- ERIC – European Research Initiative on CLL <http://ericll.org>

5. Reagents

5.1. Reagent Components

REF	5-101-0030	<i>IGH</i> Somatic Hypermutation Assay v2.0 - Gel Detection	33 Reactions
REF	5-101-0031	<i>IGH</i> Somatic Hypermutation Assay v2.0 - ABI Fluorescence Detection	33 Reactions
REF	5-101-0040	<i>IGH</i> Somatic Hypermutation Assay MegaKit v2.0 - Gel Detection	330 Reactions
REF	5-101-0041	<i>IGH</i> Somatic Hypermutation Assay MegaKit v2.0 - ABI Fluorescence Detection	330 Reactions

Table 1. Reagents for Gel Detection Assays

Reagent	Part Number	Reagent Components (active ingredients)	Unit Quantity	5-101-0030 # of Units	5-101-0040 # of Units	Storage Temp.
Master Mixes	2-101-0170	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 to -65°C
	2-101-0180	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	-85 to -65°C
Template Amplification Control Master Mix	2-096-0020	Specimen Control Size Ladder – Unlabeled Multiple oligonucleotides targeting housekeeping genes.	1500 µL	1	10	-85 to -65°C
Positive Control DNA and RNA	4-088-0730	IVS-0013 Clonal Control DNA 200 µg/mL of DNA in 1/10 th TE solution	100 µL	1	5	2 to 8°C or -85 to -65°C
	4-089-1090	IVS-0013 Clonal Control RNA 400 µg/mL of RNA in water	100 µL	1	5	-85 to -65°C
Negative (Normal) Control DNA	4-092-0010	IVS-0000 Polyclonal Control DNA 200 µg/mL of DNA in 1/10 th TE solution	100 µL	1	5	2 to 8°C or -85 to -65°C
Sequencing Primer	3-101-0380	<i>IGH</i> JH Primer – Unlabeled 100µM of DNA primer in water	10 µL	1	5	-15 to -25°C or -85 to -65°C
	3-000-0000	Primer - Hypermutation – Unlabeled 100µM of DNA primer in water	10 µL	1	5	-15 to -25°C or -85 to -65°C

Note: There are no preservatives used in the manufacture of this kit.

Table 2. Reagents for ABI Fluorescence Detection Assays

Reagent	Part Number	Reagent Components (active ingredients)	Unit Quantity	5-101-0031 # of Units	5-101-0041 # of Units	Storage Temp.
Master Mixes	2-101-0171	Hypermutation Mix 1 v2.0 – 6-FAM Multiple oligonucleotides targeting the leader region of the immunoglobulin heavy chain gene in a buffered salt solution.	1500 µL	1	10	-85 to -65°C
	2-101-0181	Hypermutation Mix 2 v2.0 – 6-FAM Multiple oligonucleotides targeting the framework 1 region of the immunoglobulin heavy chain gene in a buffered salt solution.	1500 µL	1	10	-85 to -65°C
Template Amplification Control Master Mix	2-096-0021	Specimen Control Size Ladder – 6-FAM Multiple oligonucleotides targeting housekeeping genes.	1500 µL	1	10	-85 to -65°C
Positive Control DNA and RNA	4-088-0730	IVS-0013 Clonal Control DNA 200 µg/mL of DNA in 1/10 th TE solution	100 µL	1	5	2 to 8°C or -85 to -65°C
	4-089-1090	IVS-0013 Clonal Control RNA 400 µg/mL of RNA in water	100 µL	1	5	-85 to -65°C
Negative (Normal) Control DNA	4-092-0010	IVS-0000 Polyclonal Control DNA 200 µg/mL of DNA in 1/10 th TE solution	100 µL	1	5	2 to 8°C or -85 to -65°C
Sequencing Primer	3-101-0380	IGH JH Primer – Unlabeled 100µM of DNA primer in water	10 µL	1	5	-15 to -25°C or -85 to -65°C
	3-000-0000	Primer - Hypermutation – Unlabeled 100µM of DNA primer in water	10 µL	1	5	-15 to -25°C or -85 to -65°C

Note: There are no preservatives used in the manufacture of this kit.

5.2. Warnings and Precautions

1. This product is For Research Use Only

- The assay kit should be used as a system. Do not substitute other manufacturer's reagents. Dilution, reducing amplification reaction volumes or other deviation in this protocol may affect the performance of this test and/or nullify any limited sublicense that comes with the purchase of this testing kit.
- 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. Care should be taken 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.
- Laboratory personnel are reminded to wear appropriate personal protective equipment and follow good laboratory practices and universal precautions when working with specimens. Specimens should be handled in approved biological safety containment facilities and opened only in certified biological safety cabinets. It is recommended that glass distilled de-ionized molecular biology grade water be used with the preparation of specimen DNA.
- Due to the analytical sensitivity of this test, extreme care should be taken to avoid the contamination of reagents or amplification mixtures with samples, controls or amplified materials. All reagents should be closely monitored for signs of contamination (e.g., negative controls giving positive signals). Discard reagents suspected of contamination.
- 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. Work flow in the PCR laboratory should always be in a one way direction between separate work areas; beginning in Master Mix Preparation, moving to the Specimen Preparation, then to the Amplification and finally to Detection. Do not bring amplified DNA into

the areas designated for master mix or specimen preparation.

10. All pipettes, pipette tips and any equipment used in a particular area should be dedicated to and kept to that area of the laboratory.
11. Sterile, disposable plastic ware should be used whenever possible to avoid RNase, DNase or cross-contamination.

5.3. Storage and Handling

- For any duration other than immediate use, **assay kits should be stored at -65°C to -85°C.**
- The optimum storage temperature for DNA controls is 2°C to 8°C, but DNA controls can be stored at -65°C to -85°C.
- All reagents and controls must be thawed and vortexed or mixed thoroughly prior to use to ensure that they are resuspended completely. Excessive vortexing may shear DNA and cause labeled primers to lose their fluorophors.
- Materials are stable until the labeled expiration date when stored and handled as directed. Do not use kits beyond their expiration date.
- Due to high salt concentrations, PCR master mixes are sensitive to freeze/thaw cycles. Aliquot master mixes into sterile o-ring screw-cap tubes if necessary.

6. Instruments

6.1. 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 8.4 *Amplification* for thermal cycler program.

6.2. 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 sections 8.5 *Agarose Gel Detection* and 8.6 *Polyacrylamide Gel Detection* for sample preparation.

6.3. 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.

6.4. 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.
- The ABI instrument used should be calibrated with the Dye Set(s) for 6-FAM, HEX, NED and ROX.
- Use the default settings for your polymer and capillary type.
- See section 8.8 *ABI Fluorescence Detection* for sample preparation.

7. Specimen Collection and Preparation

7.1. Precautions

Biological specimens from humans may contain potentially infectious materials. All specimens should be handled in accordance with the OSHA Standard on Bloodborne Pathogens or Biosafety Level 2.

7.2. Interfering Substances

The following substances are known to interfere with PCR:

1. Divalent cation chelators.
2. Low retention pipette tips
3. EDTA (not significant at low concentrations)
4. Heparin

7.3. Specimen Requirements and Handling

This assay tests **genomic DNA or complementary DNA (cDNA)** from the following sources:

1. 5cc of peripheral blood, bone marrow biopsy or bone marrow aspirate anti-coagulated with heparin or EDTA (stored at 2°C to 8°C and shipped at ambient temperature)
2. Minimum 5mm cube of tissue (stored and shipped frozen)
3. 2µg of genomic DNA (stored at 2°C to 8°C or -65°C to -85°C and shipped at ambient temperature)
4. 5µg of total RNA or mRNA (stored at -65°C to -85°C and shipped on dry ice)
5. 1µg of cDNA (stored at 2°C to 8°C or -65°C to -85°C and shipped at ambient temperature)
6. Formalin-fixed paraffin embedded tissue or slides (stored and shipped at ambient temperature)

7.4. Genomic DNA Sample Preparation

- This assay can be used with either genomic DNA or cDNA.

Extract the genomic DNA from patient specimens as soon as possible. Resuspend DNA to a final concentration of 100 µg to 400 µg per mL in 1/10th TE (1 mM Tris-HCl, pH 8.0; 0.1 mM EDTA) or in molecular biology grade or USP water. This is a robust assay system. A wide range of DNA concentrations will generate a valid result. Therefore, quantifying and adjusting DNA concentrations is generally not necessary. Testing sample DNA with the Specimen Control Size Ladder master mix will ensure that DNA of sufficient quality and quantity was present to yield a valid result.

7.5. Genomic DNA Sample Storage

- Genomic DNA should be stored at 2°C to 8°C or at -65°C to -85°C until use.

7.6. RNA and cDNA Sample Preparation

- This assay can be used with either genomic DNA **or** cDNA.

Extract total RNA or mRNA from patient specimens as soon as possible. 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. Synthesize cDNA from RNA. Below is a recommended protocol. Use 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):

cDNA Synthesis Protocol

1. In a single microcentrifuge tube, mix:
 - 24.0 µL of RNA
 - 10.0 µL of 5X SuperScript first-strand buffer
 - 2.0 µL of 25 mM dNTPs
 - 3.0 µL of water
 - 5.0 µL of 100mM DTT
 - 2.0 µL of random primers (3µg/µl)
 - Total volume of 46 µL.
2. Heat to 96°C to 100°C for 2 minutes and quick chill on ice.
3. Collect sample with a brief spin.
4. Add 2 µL of RNasein (5 units/µl) and mix.
5. Add 2 µL of SuperScript II and mix. (Final reaction volume of 50 µL.)
6. Place tubes at 42°C for 90 minutes. Note: it is best to 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.
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.
8. Use cDNA as is, it is not necessary to dilute further.

Testing sample cDNA with the Specimen Control Size Ladder master mix will ensure that cDNA of sufficient quality and quantity was present to yield a valid result. Please note if RNA or cDNA is contaminated with genomic DNA, the Specimen Control Size Ladder may be amplifying the genomic DNA and may produce results that are not indicative of the quality and quantity of the cDNA present. This is due to the fact that the Specimen Control Size Ladder targets single gene exons (primers do not span intron/exon borders). If possible, we suggest removing genomic DNA contamination from RNA samples with DNase, however, the DNase should be properly inactivated or removed from the sample prior to cDNA synthesis.

7.7. RNA and cDNA Sample Storage

- RNA should be stored at -65°C to -85°C until use.
- cDNA should be stored at 2°C to 8°C or at -65°C to -85°C until use.

8. Assay Procedure

8.1. Materials Provided

Table 3. Materials Provided for Gel Detection Assays

Part #	Description
2-101-0170	Hypermutation Mix 1 v2.0 - Unlabeled
2-101-0180	Hypermutation Mix 2 v2.0 – Unlabeled
2-096-0020	Specimen Control Size Ladder – Unlabeled
4-088-0730	IVS-0013 Clonal Control DNA
4-089-1090	IVS-0013 Clonal Control RNA
4-092-0010	IVS-0000 Polyclonal Control DNA
3-101-0380	<i>IGH</i> JH Primer – Unlabeled
3-000-0000	Primer – Hypermutation - Unlabeled

Table 4. Materials Provided for ABI Fluorescence Detection Assays

Part #	Description
2-101-0171	Hypermutation Mix 1 v2.0 – 6-FAM
2-101-0181	Hypermutation Mix 2 v2.0 – 6-FAM
2-096-0021	Specimen Control Size Ladder – 6-FAM
4-088-0730	IVS-0013 Clonal Control DNA
4-089-1090	IVS-0013 Clonal Control RNA
4-092-0010	IVS-0000 Polyclonal Control DNA
3-101-0380	<i>IGH</i> JH Primer – Unlabeled
3-000-0010	Primer - Hypermutation

8.2. Materials Not Provided

Table 5

Reagent/Material	Recommended Reagents/Materials and Suppliers	Notes
DNA Polymerase	Life Technologies: AmpliTaq Gold® or equivalent DNA Polymerase (Cat# N808-0241) Roche: EagleTaq or Equivalent DNA Polymerase (Cat# 05206944190)	N/A
Glass Distilled De-ionized Molecular Biology Grade or USP Water	N/A	Water should be 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	Must be able to accurately measure 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
Vortex Mixer	N/A	N/A
PCR plates or tubes	N/A	Sterile
Filter barrier pipette tips	N/A	Sterile, RNase/DNase/Pyrogen-free
Microcentrifuge tubes	N/A	Sterile

Reagent/Material	Recommended Reagents/Materials and Suppliers	Notes
Reverse Transcriptase	Invitrogen: SuperScript II (SSII) RT (Cat# 18064-071)	Includes 100mM DTT and 5X SuperScript first-strand buffer
25mM dNTP mix	Invivoscribe Technologies: 25mM dNTP mix, 0.1ml (Cat# 6-097-0020) 25mM dNTP mix, 0.5ml (Cat# 6-097-0030) 25mM dNTP mix, 1.0ml (Cat# 6-097-0040) GE Healthcare/Amersham Biosciences: Nucleotide Mix, 25mM (Cat# 25-6008-10)	N/A
Random Primers	Invitrogen: Random Primers (Cat# 48190-011)	N/A
Ribonuclease Inhibitor	Promega: RNasin Ribonuclease Inhibitor (Cat# N2115)	N/A
Gel Electrophoresis Unit	N/A	For agarose or polyacrylamide gels
Agarose	Cambrex: MetaPhor® Agarose (Cat# 50180) NuSieve® 3:1 Agarose (Cat# 50090)	N/A
Ethidium Bromide	Invitrogen: UltraPure™ 10 mg/mL Ethidium Bromide (Cat# 15595-011)	N/A
6% Polyacrylamide Gels	Invitrogen: Novex® TBE Gels (6%, 12 well) (Cat# EC62652Box)	N/A
TBE Running Buffer	Invitrogen: Novex® TBE Running Buffer (5X) (Cat# LC6675)	Dilute 1:5 prior to use.
Gel Loading Buffer	Invivoscribe Technologies: 6X Loading Buffer (No Dyes) (Cat# 6-098-0010) 6X Loading Dye I (Light Dyes) (Cat# 6-098-0020) 6X Loading Dye II (Very Light Dyes) (Cat# 6-098-0030) Invitrogen: 10X BlueJuice™ Gel Loading Buffer (Cat# 10816-015) Novex® Hi-Density TBE Buffer (5X) (Cat# LC6678)	N/A
100 bp DNA Ladder	Invitrogen: TrackIt™ 100 bp DNA Ladder (Cat# 10488-058)	N/A
ABI Capillary Electrophoresis Instrument	Applied Biosystems: ABI 310, 3100 or 3130 series	N/A
Hi-Di Formamide	Invivoscribe Technologies: HI-Deionized Formamide (Cat# 6-098-0041) Applied Biosystems: Hi-Di™ Formamide (Cat# 4311320)	N/A
ROX Size Standards	Invivoscribe Technologies: Hi-Di Formamide w/ROX size standards for ABI 310 (Cat# 6-098-0051) Hi-Di Formamide w/ROX size standards for ABI 3100 (Cat# 6-098-0061) Applied Biosystems: GeneScan™ - 400HD [ROX]™ (Cat# 402985)	N/A
Spectral Calibration Dye Set D	Applied Biosystems: For ABI 3100 and 3130 instruments: DS-30 Matrix Standard Kit (Dye Set D) (Cat# 4345827) For ABI 310 instruments: NED Matrix Standard (Cat# 402996) And Fluorescent Amidite Matrix Standards [6FAM, TET, HEX, TAMRA, ROX] (Cat# 401546)	Dye set used to spectrally calibrate ABI instrument for use with 6-FAM, HEX, NED and ROX

Reagent/Material	Recommended Reagents/Materials and Suppliers	Notes
Polymer	Applied Biosystems: POP-4 Polymer: POP-4™ for 310 Genetic Analyzers (Cat# 402838) POP-4™ for 3100/3100-Avant Genetic Analyzers (Cat# 4316355) POP-4™ for 3130/3130xl Genetic Analyzers (Cat# 4352755) POP-7 Polymer: POP-7™ for 3130/3130xl Genetic Analyzers (Cat# 4352759)	N/A
Buffer	Applied Biosystems: 10X Genetic Analyzer Buffer with EDTA (Cat# 402824)	Dilute 1:10 in sterile water before use
Gel Extraction Kit	Qiagen: MinElute Gel Extraction Kit (Cat# 28604) QIAEX II Gel Extraction Kit (Cat# 20021)	N/A
TA Cloning Kit	Invitrogen: TOPO TA Cloning® Kit (with pCR®2.1-TOPO® vector) with OneShot TOP10 Chemically Competent E. Coli (Cat# K4500-01)	N/A
Plasmid Isolation Kit	Qiagen: QIAprep Spin MiniPrep Kit (Cat# 27104)	N/A

8.3. Reagent Preparation

- All unknown samples should be tested using the Specimen Control Size Ladder master mix. This is to ensure that no inhibitors of amplification are present and there is genomic DNA or cDNA of sufficient quality and quantity to generate a valid result.
 - **Positive, negative and no template** controls should be tested for each of the master mixes.
1. Using gloved hands, remove the master mixes from the freezer. Allow the tubes to thaw completely; then gently vortex to mix.

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

Aliquot volumes should be 45 µL for each reaction.

We recommend adding an additional reaction for every 15 reactions to correct for pipetting errors. Thus, for each master mix (except for the Specimen Control Size Ladder), the number of reactions (**n**) should be:

n = # of samples	(run each sample in singlicate)
+ 1	positive control DNA (required if testing genomic DNA 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
<hr/>	
n = # of samples + 5	Total

Therefore the total aliquot volume for each master mix should be **n** × 45 µL.

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

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
<hr/>	
m = # of samples + 3	Total

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

Add 1.25 units (or 0.25 µL @5 U/µL) of AmpliTaq Gold or EagleTaq DNA polymerase per reaction to each master mix. The total AmpliTaq Gold or EagleTaq DNA polymerase added to each master mix should be **n** × 0.25 µL and **m** × 0.25 µL for the Specimen Control Size Ladder master mix. Gently vortex to mix.

- For each reaction, aliquot 45 µL of the appropriate master mix + DNA polymerase solution into individual wells in a PCR plate or tube.
- Add 5 µL of appropriate template (sample genomic DNA 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.
- Cap or cover the PCR plate.
- Samples are now ready to be amplified on a thermal cycler.
 - If amplification cannot be performed immediately following reagent preparation, the PCR plate or tubes can be stored at 2°C to 8°C for up to 24 hours.

Quick Guide:

For each master mix and **n** reactions, mix:

n × 45 µL Master Mix

n × 0.25 µL AmpliTaq Gold or EagleTaq 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**

8.4. Amplification

- Amplify the samples using the following PCR program:

(Note: We recommend using the **calculated** option for temperature measurement with the BioRad MJ Research PTC thermal cyclers.)

Standard Program for AmpliTaq Gold or EagleTaq

Step 1: 95°C for 7 minutes

Step 2: 95°C for 45 seconds

Step 3: 60°C for 45 seconds

Step 4: 72°C for 90 seconds

Step 5: Go to step 2; 34 more times

Step 6: 72°C for 10 minutes

Step 7: 15°C forever

- Remove the amplification plate or tubes from the thermal cycler.
- Proceed with one of the following detection methods in sections 8.5 through 8.8.
 - Although amplified DNA is stable at room temperature for extended periods of time, PCR products should be stored at 2°C to 8°C until detection. Detection must be within 30 days of amplification.

8.5. Agarose Gel Detection

- This section is for detection of samples amplified with Unlabeled Master Mixes.
- 1. Prepare a 2% MetaPhor or NuSieve 3:1 agarose/1X TBE gel with large combs.
- 2. Place gel in electrophoresis unit and cover with 1X TBE buffer.
- 3. Mix 20 μ L of each PCR product with 4 μ L of 6X gel loading buffer.
- 4. Load 20 μ L of this mixture into separate wells of the gel and 4 μ L of the 100 bp DNA Ladder flanking the samples.
- 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.
- 6. Stain gel with ethidium bromide or other dye.
- 7. Place gel over UV illuminator and photograph data.
- 8. Interpret data. (See section 9 *Band Interpretation and Expected Values.*)
- 9. Proceed with sequencing. (See section 10 *Sequencing of PCR Product.*)

8.6. Polyacrylamide Gel Detection

- This section is for detection of samples amplified with Unlabeled Master Mixes.
- 1. Assemble the electrophoresis unit using a non-denaturing, 6% polyacrylamide TBE gel and 1X TBE running buffer.
- 2. Mix 20 μ L of each sample with 5 μ L of ice-cold non-denaturing 5X bromophenol blue loading buffer.
- 3. Load all 20 μ L of the mixture into individual wells of the gel.
- 4. Run gel at 110V 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.
- 5. Stain gels in 0.5 μ g/ml ethidium bromide (in water or 0.5X TBE Buffer) for 5-10 minutes.
- 6. De-stain gels in water for 5-10 minutes. Repeat with new water.
- 7. Place gel over UV illuminator and photograph data.
- 8. Interpret data. (See section 9 *Band Interpretation and Expected Values.*)
- 9. Proceed with sequencing. (See section 10 *Sequencing of PCR Product.*)

8.7. Heteroduplex Analysis

- This section is for detection of samples amplified with Unlabeled Master Mixes.
- 1. Denature 20 μ L of PCR products at 94°C for 5 minutes.
- 2. Quick chill PCR products at 4°C (on an ice water bath) for 60 minutes.
- 3. Proceed with section 8.6 *Polyacrylamide Gel Detection*, above.

8.8. ABI Fluorescence Detection

- Please note that for ABI fluorescence detection a preceding peak is often seen and is an artifact due to the detection method the ABI platforms use. Preceding peaks are sometimes skewed and have bases that slope on the right side towards the real peak. This is especially evident in the Specimen Control Size Ladder master mix where the 96 nucleotide peak has a preceding peak that shows up at 84 nucleotides.
1. In a new microcentrifuge tube, mix an appropriate amount (for a total of 10 μ L per reaction) of Hi-Di Formamide with ROX Size Standards^a. Vortex well.
 2. In a new 96-well PCR plate, add 10 μ L of Hi-Di Formamide with ROX size standards to individual wells for each reaction.
 3. Transfer 1 μ L of each reaction to the wells containing Hi-Di Formamide with ROX size standards. Add only one sample per well. Pipette up and down to mix.
 4. Cap or cover the PCR plate or tubes.
 5. Heat denature the samples at 95°C for 2 minutes then snap chill on ice for 5 minutes.
 6. Prepare a **sample sheet** and **injection list** for the samples.
 7. Run the samples on an ABI capillary electrophoresis instrument according to the user manual^b.
 8. Data are automatically displayed as size and color specific peaks. Review profile and controls, report results. (See section 9 *Band Interpretation and Expected Values*.)
 9. Proceed with sequencing. (See section 10 *Sequencing of PCR Product*.)

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

Note^b: As the samples are run on the machine, they are fractionated, detected and analyzed by the instrument. Runs are 20-24 minutes in duration. The ABI capillary electrophoresis instruments routinely handle 2 runs per hour (for the 1-, 4- and 16-capillary instruments this is equal to 48, 192 and 768 samples per day, respectively) and automatically analyze and store data for review or comparison with other test results.

8.9. Quality Control

Positive and negative (or normal) controls are furnished with the kit and should be run in singlicate each time the assay is performed to ensure proper performance of the assay. In addition a no template control (e.g. water) should also be included to test for contamination of the master mix or cross-contamination of 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 8*. Additional controls and sensitivity controls (dilutions of positive controls into our negative control) are available from Invivoscribe Technologies.

8.10. Recommended Positive Controls

- The amplicon sizes listed were determined using an ABI 3130XL platform. Amplicon sizes seen on your specific capillary electrophoresis instrument may differ 1 to 4 nucleotides (nt) from those listed depending on the platform of detection and the version of the analysis software used. Once identified, the amplicon size as determined on your specific platform will be consistent from run to run. This reproducibility is extremely useful when monitoring disease recurrence.
- **Note:** "Color" indicates the color of products generated with the master mix when using the default color assignment on ABI fluorescence detection systems.

Table 6

Master Mix	Target	Color	Control DNA or RNA	Cat# for DNA	Product Size (bp) for DNA	Cat# for RNA	Product Size (bp) for cDNA
Hypermethylation Mix 1 v2.0	Leader to JH	Blue	Valid Size Range IVS-0013 Clonal Control	--- 4-088-0730	500-570 536	--- 4-089-1090	415-485 461
Hypermethylation Mix 2 v2.0	FR1 to JH	Blue	Valid Size Range IVS-0013 Clonal Control	--- 4-088-0730	310-380 358	--- 4-089-1090	310-380 Same as DNA
Specimen Control Size Ladder	Multiple Genes	Blue	Valid Size Range IVS-0000 Polyclonal Control	--- 4-092-0010	96, 199, 299, 399, 608 96, 199, 299, 399, 608	--- not available	96, 199, 299, 399, 608 Same as DNA

Note: Because smaller PCR fragments are preferentially amplified, it is not unusual for the 608bp fragment for the Specimen Control Size Ladder to have a diminished signal or to be missing entirely. For ABI fluorescence detection the 608nt peak may not appear during normal run times. In addition, the size of this peak may differ by over 30nt when the fragment size is extrapolated using the GeneScan - 400HD [ROX] size standards.

9. Band Interpretation and Expected Values

Although positive results are highly suggestive of malignancy, both positive and negative results should be interpreted in the context of all clinical information and laboratory test results. The size range for each of the master mixes has been determined by testing positive and negative control samples. For accurate and meaningful interpretation it is important to ignore peaks that occur outside of the valid size range for each of the master mixes.

9.1. Analysis

1. Samples that fail to amplify following repeat testing should be reported as **“A result cannot be reported on this specimen because there was DNA of insufficient quantity or quality for analysis”**.
2. Samples that test negative should be repeated if the positive control reaction failed.
3. If samples run in duplicate yield differing results, the samples should be re-tested and/or re-evaluated for sample switching.
4. **All assay controls must be examined prior to interpretation of sample results. If the controls do not yield the correct results, the assay is not valid and the samples should not be interpreted.**

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

Table 7

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 <i>Table 8</i> . 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 <i>Table 8</i> . Continue with analysis.	Repeat the assay.
Specimen Control Size Ladder (This amplification control is <u>essential</u> for samples of unknown quantity and quality.)	If all of the 96, 199, 299, 399 and 608nt peaks are seen, continue with analysis. Because smaller PCR fragments are preferentially amplified, it is not unusual for the 608nt fragment to have a diminished signal or to be missing entirely. Continue with analysis.	If no bands are seen, repeat the assay <u>unless specimen tests positive</u> . If only 1, 2 or 3 bands are seen, re-evaluate sample for DNA degradation <u>unless specimen tests positive</u> .

9.2. **Sample Interpretation**

Given that the controls produce expected results, the samples should be interpreted as follows:

- 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. In the context of overall diagnostic criteria, clonal cell populations can indicate the presence of hematologic malignancy.
- 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).

Note ^a: Criteria for defining a positive peak are as follows:

- **For 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.
- **For 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.

Positive samples should be (amplified with the unlabeled master mixes for gel detection and run on a gel, if ABI Fluorescence detection was initially used) gel extracted and sequenced. (See section 10 *Sequencing of PCR Product*.)

9.3. **Expected Size of Amplified Products**

- The amplicon sizes listed were determined using an ABI 3130XL platform. Amplicon sizes seen on your specific capillary electrophoresis instrument may differ 1 to 4 nucleotides (nt) from those listed depending on the platform of detection and the version of the analysis software used. Once identified, the amplicon size as determined on your specific platform will be consistent from run to run. This reproducibility is extremely useful when monitoring disease recurrence.
- **Note:** “Color” indicates the color of products generated with the master mix when using the default color assignment on ABI fluorescence detection systems.

Table 8

Master Mix	Target	Color	Control DNA or RNA	Cat# for DNA	Product Size (bp) for DNA	Cat# for RNA	Product Size (bp) for cDNA
Hypermutation Mix 1 v2.0	Leader to JH	Blue	Valid Size Range IVS-0000 Polyclonal Control IVS-0013 Clonal Control	--- 4-092-0010 4-088-0730	500-570 500-570 536	--- not available 4-089-1090	415-485 415-485 461
Hypermutation Mix 2 v2.0	FR1 to JH	Blue	Valid Size Range IVS-0000 Polyclonal Control IVS-0013 Clonal Control	--- 4-092-0010 4-088-0730	310-380 310-380 358	--- not available 4-089-1090	Same as DNA
Specimen Control Size Ladder	Multiple Genes	Blue	Any Human DNA or cDNA	---	96, 200, 300, 400, 600	---	Same as DNA

Note: Because smaller PCR fragments are preferentially amplified, it is not unusual for the 608bp fragment for the Specimen Control Size Ladder to have a diminished signal or to be missing entirely. For ABI fluorescence detection the 608nt peak may not appear during normal run times. In addition, the size of this peak may differ by over 30nt when the fragment size is extrapolated using the GeneScan - 400HD [ROX] size standards.

9.4. Sample Gel Data

The data shown in Figures 2 and 3 were generated using the master mixes indicated.

- Lane 1 displays data generated testing cDNA synthesized from the recommended 100% clonal control RNA.
- Lane 2 displays data generated testing the recommended 100% clonal control DNA.
- Lane 3 displays data generated testing a 10% dilution of the recommended clonal control DNA diluted into IVS-0000 DNA.
- Lane 4 displays data generated testing IVS-0000 Polyclonal Control DNA.



Figure 2. Amplified products were generated using Hypermutation Mix 1 v2.0 then run on 2% Metaphor agarose/TBE gel.

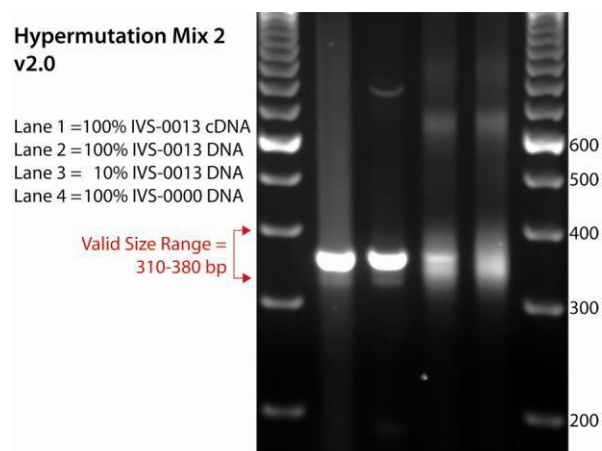


Figure 3. Amplified products were generated using Hypermutation Mix 2 v2.0 then run on 2% Metaphor agarose/TBE gel.

9.5. Sample ABI Fluorescence Detection Data

The data shown in Figures 4 and 5 were generated using the master mixes indicated.

- Panel 1 displays data generated testing cDNA synthesized from the recommended 100% clonal control RNA.
- Panel 2 displays data generated testing the recommended 100% clonal control DNA.
- Panel 3 displays data generated testing a 10% dilution of the recommended clonal control DNA diluted into IVS-0000 DNA.
- Panel 4 displays data generated testing IVS-0000 Polyclonal Control DNA

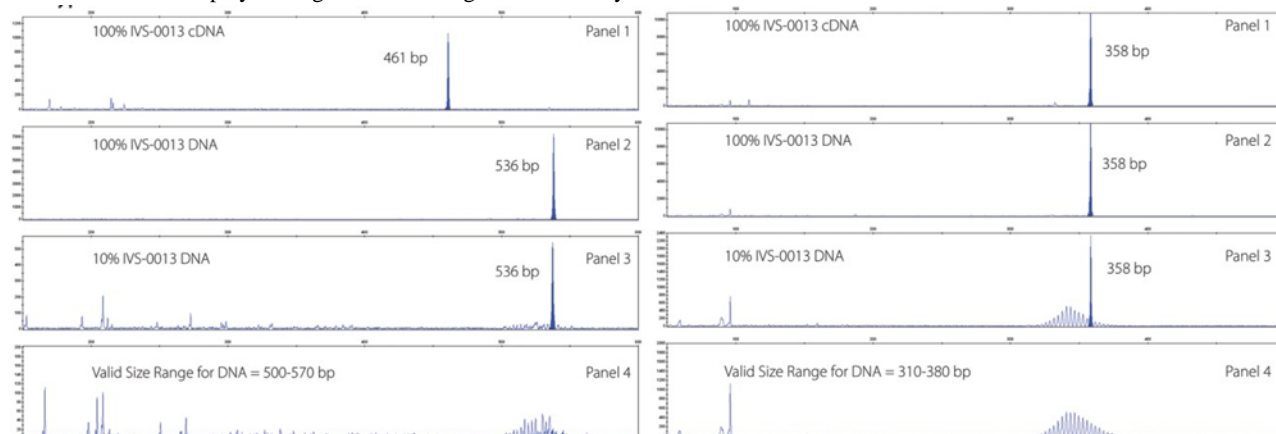


Figure 4. Amplified products were generated using Hypermutation Mix 1 v2.0 – 6FAM with genomic DNA then run on ABI 3130 instrument. .

Figure 5. Amplified products were generated using Hypermutation Mix 2 v2.0 – 6FAM with cDNA then run on ABI 3130 instrument. .

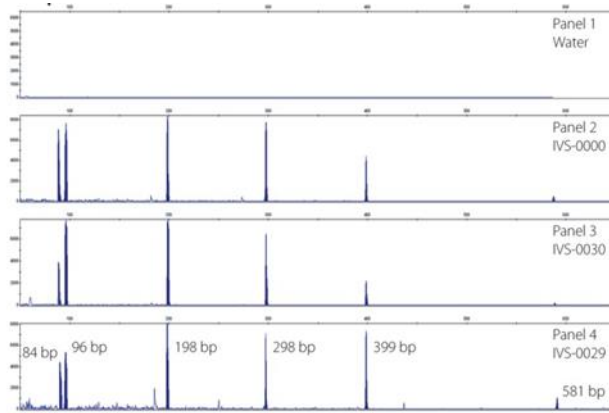


Figure 6. The Specimen Control Size Ladder master mix:

- Panel 1 displays data generated testing a negative water control.
- Panel 2 displays data generated testing the recommended positive control, IVS-0000 Polyclonal Control DNA.
- Panels 3 and 4 display data generated testing two different 100% clonal control DNAs.

10. Sequencing of PCR Product

10.1. Direct Sequencing

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

1. In two separate containers mix the appropriate concentration of the reverse sequencing primer, *IGH JH* Primer – Unlabeled and Primer – Hypermutation – Unlabeled with the PCR product.
2. Sequence the sample.
3. Proceed with section *11 Hypermutation Analysis and Reporting*.

10.2. Gel Extraction

Gel extraction should be used with weak clonal bands in a polyclonal background or if there is more than one clonal band.

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.
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.
3. Use a gel extraction kit, such as Qiagen's MinElute Gel Extraction Kit for agarose gels or Qiagen's QIAEX II Gel Extraction Kit for polyacrylamide gels, extract and elute the DNA in an appropriate buffer at a high concentration.
4. Proceed with **either** *10.1 Direct Sequencing* above **or** *10.3 Cloning and Sequencing* below.

10.3. Cloning and Sequencing

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

1. Use 4µl of the band extracted PCR product for the ligation reaction with the pCR®2.1®-TOPO vector.
2. Follow Invitrogen's protocol for transforming and plating bacteria.
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.
4. Spin each bacterial culture down at 6000 rpm in a centrifuge and pour off media.
5. Proceed with a plasmid DNA extraction. We recommend Qiagen's QIAprep Spin Miniprep Kit.
6. Sequence each plasmid sample with M13 forward and/or M13 reverse sequencing primers.
7. Proceed with section 11 *Hypermutation Analysis and Reporting*.

11. Hypermutation Analysis and Reporting

1. For sequences obtained by cloning, ignore vector sequences. Align multiple sequence data obtained from a given sample and check for concordance. The majority of the sequences should be identical. For sequences obtained by direct sequencing, adjust sequences so that they are in the V to J orientation instead of J to V.
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:

- IMGT – The International ImMunoGeneTics information system (Initiator and coordinator: Marie-Paule Lefranc, Montpellier, France) <http://imgt.cines.fr>
Analysis tools: IMGT/V-QUEST and IMGT/Junction Analysis
- V BASE – The MRC Centre for Protein Engineering's Database of human antibody genes <http://vbase.mrc-cpe.cam.ac.uk/> and <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.

3. After identifying a valid sequence, *IGH* somatic hypermutation analysis can be done using one of the listed websites. Alternatively this can be done 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.
4. Find the germline V region sequence that best corresponds to the sample sequence.
5. Align the germline V region sequence to the V region sequence of the sample.
6. Determine the number of mismatched bases and the total number of bases that are being compared.
7. The % divergence = number of mismatched bases ÷ total number of bases compared and the % homology = 100% - % divergence.
8. Complete these steps for both the forward and reverse sequences.
9. If the forward and reverse sequences are discordant for mutational status, repeat the experiment.

11.1. IVS-0013 Clonal Control Data

Sequence data obtained from the positive IVS-0013 Clonal Control DNA or RNA should correspond to an unmutated, in-frame VH1-46 to JH4 rearrangement.

11.2. Sample Data

The following is an example of a sequence alignment using V BASE's DNAPLOT analysis tool and the sequence data of a patient sample. The patient 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 VH1-02. The % divergence of this sample = $21 \div 206 = 10.2\%$.

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	-	VHGL1.2	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																					
EMBL	Locus	Name	22	23	24	25	26	27	28	29	30	31	31a	31b	32	33	34	35	36	37	38	39	40	
Z14071	1-02	DP-75/VI-2...+	TGC	AAG	GCT	TCT	GGA	TAC	ACC	TTC	ACC	-G-	TAC	TAT	TTG	CAC	TGG	GTG	CGA	CAG	GCC
X92208	1-02	1-1+	TGC	AAG	GCT	TCT	GGA	TAC	ACC	TTC	ACC	-G-
Z12310	1-02	DP-8+	TGC	AAG	GCT	TCT	GGA	TAC	ACC	TTC	ACC	-G-
Z14213	-	VHGL1.2	TGC	AAG	GCA	TCT	GGA	TAC	ACC	TTC	ACC	AG-
X07448	1-02	V35/VI-2b+	TGC	AAG	GCT	TCT	GGA	TAC	ACC	TTC	ACC	-G-
			H2																					
EMBL	Locus	Name	41	42	43	44	45	46	47	48	49	50	51	52	52a	52b	52c	53	54	55	56	57	58	
Z14071	1-02	DP-75/VI-2...+	CCT	GGA	CAA	GGG	TTT	GAG	TGG	ATG	GGA	TGG	ATC	AAC	CTT	AAG	AAT	GGT	GCC	ATC	AGG	
X92208	1-02	1-1+	---	---	---	---	C--	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	
Z12310	1-02	DP-8+	---	---	---	---	C--	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	
Z14213	-	VHGL1.2	---	---	---	---	C--	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	
X07448	1-02	V35/VI-2b+	---	---	---	---	C--	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	
			CDR2																					
EMBL	Locus	Name	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	
Z14071	1-02	DP-75/VI-2...+	TAT	GCA	CAG	AGG	TTT	GAG	GAC	AGG	GTC	ACC	ATG	ACC	AGT	GAC	ACG	TCC	ATT	AGC	ACA	GCC	TAC	
X92208	1-02	1-1+	---	---	---	-A-	---	C--	-G-	---	---	---	---	---	---	---	---	---	---	---	---	---	---	
Z12310	1-02	DP-8+	---	---	---	-A-	---	C--	-G-	---	---	---	---	---	---	---	---	---	---	---	---	---	---	
Z14213	-	VHGL1.2	---	---	---	-A-	---	C--	-G-	---	---	---	---	---	---	---	---	---	---	---	---	---	---	
X07448	1-02	V35/VI-2b+	---	---	---	-A-	---	C--	-G-	---	---	---	---	---	---	---	---	---	---	---	---	---	---	
			CDR3																					
EMBL	Locus	Name	80	81	82	82a	82b	82c	83	84	85	86	87	88	89	90	91	92	93	94	95			
Z14071	1-02	DP-75/VI-2...+	ATG	GAG	CTG	GGC	AGG	CTG	AAA	TCA	GAC	GAC	ACG	GCT	GTA	TAT	TAC	TGT	GCG	AGA	GAG			
X92208	1-02	1-1+	---	---	---	A--	---	---	-G-	---	---	---	---	---	---	---	---	---	---	---	---			
Z12310	1-02	DP-8+	---	---	---	A--	---	---	-G-	---	---	---	---	---	---	---	---	---	---	---	---			
Z14213	-	VHGL1.2	G--	---	---	A--	---	---	-G-	---	---	---	---	---	---	---	---	---	---	---	---			
X07448	1-02	V35/VI-2b+	---	---	---	A--	---	---	-G-	---	---	---	---	---	---	---	---	---	---	---	---			

The following is the same sequence analyzed with NCBI's 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 VH1-02 (additional sequences were listed, but only the top three matches are shown below). IgBlast provides the percentage of identity (or homology) given by (ID%). For this sample, it is 90% homologous to the germline VH1-02, therefore the % divergence = $100\% - 90\% = 10\%$.

ID%	tmpseq	3	<-----CDR1----->	<-----FWR2----->	<----->
90	VH1-2	91	--CTACTATTTCAC	TGGGTGCGACAGGCCCTGGACAAAGGGTTTGAGTGGATGGGA	TGGATCA-ACCTTAAGAATGGTG 79
86	VH1-8	104	GG.....A.....C.....C..C.G.....169
84	VH1-46	93	-----A.....C.....	ATA.....C..GTGG...A 169
ID%	tmpseq	80	<-----CDR2----->	<-----FWR----->	<----->
90	VH1-2	170	CCATCAGG-TATGCACAGAGGTTTGAGGAC	AGGGTCACCATGACCACTGACACGTCCTATTAGCACAGCCTACATGGAGCT 158	
86	VH1-8	170	G..CA.AC.....A.....C..G.G.....C.....C..C.G.....248
84	VH1-46	170	A.....C.....A.....CC..G.A.....GA.....C.....A.....248
			G..CA..C-..C.....A.....CC..G.A.....G.....CG.....T.....248
ID%	tmpseq	159	3----->		
90	VH1-2	249	GGGACGGGTGAAATCAGACGACACGGCTGTATATTACTGTGCGAGA	204	
86	VH1-8	249	.A.....G...T.....C..G.....	294	
84	VH1-46	249	.A.....C.....G...T..G.....C..G.....	294	
			.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 VH1-02 (additional sequences are listed). The % divergence = $25 \div 231 = 10.8\%$.

```

<----- F R 1 - I M G T ----->
      1           5           10          15
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

----->
      20           25           30           35
input
X62106 IGHV1-2*02 ... .. AGA AAC ACA TTC AGC GAC TAC TAT ... ..
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 -G- -- --

<----- F R 2 - I M G T ----->
      40           45           50
input
X62106 IGHV1-2*02 ... .. TTG CAC TGG GTG CGA CAG GCC CCT GGA CAA GGG TTT GAG TGG ATG GGA
X92208 IGHV1-2*03 ... .. A-- -- -- -- -- -- -- -- -- -- -- -- -- -- -- -- -- -- -- --
Z12310 IGHV1-2*04 ... .. A-- -- -- -- -- -- -- -- -- -- -- -- -- -- -- -- -- -- -- --
X07448 IGHV1-2*01 ... .. A-- -- -- -- -- -- -- -- -- -- -- -- -- -- -- -- -- -- -- --
L06612 IGHV1-46*03 ... .. A-- -- -- -- -- -- -- -- -- -- -- -- -- -- -- -- -- -- -- --

-->
      55           60           65           70
input
X62106 IGHV1-2*02 TGG ATC AAC CTT AAG AAT GGT GCC ATC ... .. AGG TAT GCA CAG AGG TTT GAG
X92208 IGHV1-2*03 --- -- -- --C --G-- --G-- --CA ... .. -AC -- -- -- --A-- --C--
Z12310 IGHV1-2*04 --- -- -- --C --G-- --G-- --CA ... .. -AC -- -- -- --A-- --C--
X07448 IGHV1-2*01 --- -- -- --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 ----->
      75           80           85           90
input
X62106 IGHV1-2*02 ... GAC AGG GTC ACC ATG ACC AGT GAC ACG TCC ATT AGC ACA GCC TAC ATG GAG
X92208 IGHV1-2*03 ... -G- -- -- -- -- -- -- --G -- -- --C -- -- -- -- -- -- --
Z12310 IGHV1-2*04 ... -G- T-- -- -- -- -- -- --G -- -- --C -- -- -- -- -- -- --
X07448 IGHV1-2*01 ... -G- T-- -- -- -- -- -- --G -- -- --C -- -- -- -- -- -- --
L06612 IGHV1-46*03 ... -G- --A -- -- -- -- -- -- --G -- -- --CG -- -- -- --T-- -- -- --

----->
      95           100          105
input
X62106 IGHV1-2*02 CTG GGC AGG CTG AAA TCA GAC GAC ACG GCT GTA TAT TAC TGT GCG AGA
X92208 IGHV1-2*03 --- A-- -- -- --G-- --T -- -- -- --C --G -- -- --TGT -- -- --
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. Limitations of Procedure

- This assay does not identify 100% of clonal cell populations.
- This assay cannot reliably detect less than 5 positive cells per 100 normal cells.
- The results of molecular clonality tests should always be interpreted in the context of clinical, histological and immunophenotypic data.
- PCR-based assays are subject to interference by degradation of DNA or to inhibition of PCR due to EDTA, heparin and other agents.

13. Bibliography

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14. Technical and Customer Service

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16. Symbols

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

	Catalog Number		Expiration Date
	Reagent Volume		Authorized Representative in the European Community
	Lot Number		Consult Instructions for Use
	Storage Conditions		For <i>In Vitro Diagnostic</i> Use
	Manufacturer		