Instructions for Use

TCRB Gene Clonality Assay

RUO

For identification of clonal T-cell receptor beta chain gene rearrangements.

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

Manufactured in U.S.A.

invivoscribe

Storage Conditions: -85ºC to -65ºC
(DNA controls may be separated from assay kits and stored at 2°C to 8°C)

Catalog#		Products	Quantity
REF	12050010	*TCRB* Gene Clonality Assay for Gel Detection	33 Reactions
REF	12050011	*TCRB* Gene Clonality Assay for ABI Fluorescence Detection	33 Reactions
REF	12050020	*TCRB* Gene Clonality Assay MegaKit for Gel Detection	330 Reactions
REF	12050021	*TCRB* Gene Clonality Assay MegaKit for ABI Fluorescence Detection	330 Reactions

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1. Assay Use

This Research Use Only assay uses PCR to detect clonal T-cell receptor beta chain gene rearrangements and is useful for the study of clonal T-cell populations and evaluation of new research and methods in malignancy studies.

2. Summary and Explanation of the Test

2.1. Background

Rearrangements of the antigen receptor genes occur during ontogeny in B and T lymphocytes. These gene rearrangements generate products that are unique in length and sequence for each cell. Therefore, polymerase chain reaction (PCR) assays can be used to identify lymphocyte populations derived from a single cell by detecting the unique V-J gene rearrangements present within these antigen receptor loci. The human TCR beta gene locus on chromosome 7 (7q35) includes 64-67 V genes belonging to 32 subgroups, 2 D segments and 13 J segments, spread over 620 kilobases. This assay employs multiple consensus DNA primers that target conserved genetic regions within the T-cell receptor beta chain gene. This test is used to detect the vast majority of clonal T-cell malignancies from DNA. Test products can be analyzed using a variety of detection formats, including gel and capillary electrophoresis.

2.2. Summary

Invivoscribe's gene clonality and translocation assays represent a simple approach to PCR-based clonality testing. These standardized assays were carefully optimized testing positive and negative control samples using multiplex master mixes. This assay kit includes three (3) multiplex master mixes that target conserved regions within the variable (V), diversity (D) and the joining (J) regions that flank the unique hypervariable antigen-binding region 3 (CDR3). Tube A contains 23 Vβ primers + 6 Jβ1 primers + 3 Jβ2 primers. Tube B contains 23 Vβ + 4 Jβ2 primers. Tube C contains 2 Dβ + 13 Jβ primers (Figure 1). Positive and negative DNA controls, as well as an internal Specimen Control Size Ladder master mix are included. PCR products can be analyzed by differential fluorescence detection using capillary electrophoresis or gene sequencing instruments, by heteroduplex analysis or using standard gel electrophoresis with ethidium staining. Clonality is indicated if any one of the master mixes generates clonal band(s).

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

EuroClonality

3. Principles of the Procedure

3.1. Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) assays are routinely used for the identification of clonal B- and T-cell populations. These tests amplify the DNA between primers that target the conserved framework (FR) and joining (J) regions (Tubes A and B) or the diversity and joining regions (Tube C). These conserved regions lie on either side of an area within the V–J region where programmed genetic rearrangements occur during maturation of all B and T lymphocytes. The antigen receptor genes that undergo rearrangement are the immunoglobulin heavy and light chains in B-cells and the T-cell receptor genes in T-cells. Each B- and T-cell has a single productive V-J rearrangement that is unique in both length and sequence. Therefore, when this region is amplified using DNA primers that flank this region, a clonal population of cells yields one or two prominent amplified products (amplicons) within the expected size range. Two products are generated in cases when the initial rearrangement was non-productive and followed by rearrangement of the other homologous chromosome. In contrast, DNA from a normal or polyclonal (many clones) population produces a bell-shaped curve of amplicon products (or Gaussian distribution) that reflect the heterogeneous population of V-J region rearrangements.

Since the antigen receptor genes are polymorphic (consisting of a heterogeneous population of related DNA sequences), it is difficult to employ a single set of DNA primer sequences to target all of the conserved flanking regions around the V-J rearrangement. N-region diversity and somatic mutation further scramble the DNA sequences in these regions. Therefore, multiplex master mixes, which target several FR regions, are required to identify the majority of clonal rearrangements. As indicated, clonal rearrangements are identified as prominent, single-sized products within

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

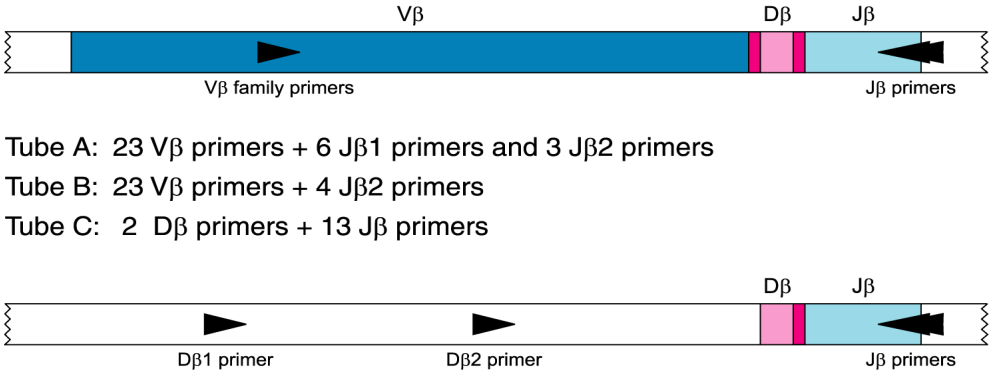


Figure 1. This is a simplified diagram of a representative rearranged T-cell receptor beta gene on chromosome 7 (7q35) showing the approximate placement of the upstream and downstream DNA primers. The numbers of primers and their specificity are listed for master mix Tubes A, B, and C.

3.2. Gel 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 dyes can then be used to stain and detect these products in the gel.

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

3.3. Differential Fluorescence Detection

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.

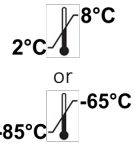
4. Reagents

4.1. Reagent Components

Table 1. Available Kits

	Catalog #	Description	Quantity
REF	12050010	*TCRB* Gene Clonality Assay for Gel Detection	33 Reactions
REF	12050011	*TCRB* Gene Clonality Assay for ABI Fluorescence Detection	33 Reactions
REF	12050020	*TCRB* Gene Clonality Assay MegaKit for Gel Detection	330 Reactions
REF	12050021	*TCRB* Gene Clonality Assay MegaKit for ABI Fluorescence Detection	330 Reactions

Table 2. Reagent Components

Reagent	Catalog #	Reagent Components (active ingredients)	Unit Quantity	Assay Kit # of Units	Assay MegaKit # of Units	Storage Temp.
Positive Control DNA	40880490	**IVS-0009 Clonal Control DNA** 200 µg/mL of DNA in 1/10th TE solution	100 µL	1	5	
	40880190	**IVS-0004 Clonal Control DNA** 200 µg/mL of DNA in 1/10th TE solution	100 µL	1	5	
	40881210	**IVS-0021 Clonal Control DNA** 200 µg/mL of DNA in 1/10th TE solution	100 µL	1	5	
Negative (Normal) Control DNA	40920010	**IVS-0000 Polyclonal Control DNA** 200 µg/mL of DNA zin 1/10th TE solution	100 µL	1	5	
Gel Detection						
Master Mixes	22050010	***TCRB* Tube A - Unlabeled** Multiple oligonucleotides targeting the Vβ + Jβ1 + Jβ2 regions of the T-cell receptor beta gene in a buffered salt solution.	1500 µL	1	10	
	22050020	***TCRB* Tube B - Unlabeled** Multiple oligonucleotides targeting the Vβ + Jβ2 regions of the T-cell receptor beta gene in a buffered salt solution.	1500 µL	1	10	
	22050030	***TCRB* Tube C - Unlabeled** Multiple oligonucleotides targeting the Dβ + Jβ1 + Jβ2 regions of the T-cell receptor beta 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	
Differential Fluorescence Detection						
Master Mixes	22050011	***TCRB* Tube A - 6FAM & HEX** Multiple oligonucleotides targeting the Vβ + Jβ1 + Jβ2 regions of the T cell receptor beta gene in a buffered salt solution.	1500 µL	1	10	
	22050021	***TCRB* Tube B - 6FAM** Multiple oligonucleotides targeting the Vβ + Jβ2 regions of the T-cell receptor beta gene in a buffered salt solution.	1500 µL	1	10	
	22050031	***TCRB* Tube C - 6FAM & HEX** Multiple oligonucleotides targeting the Dβ + Jβ1 + Jβ2 regions of the T-cell receptor beta 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	

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

4.2. Warnings and Precautions

- RUO **This product is for Research Use Only; not for use in diagnostic procedures**.
- Use this assay kit as a system; do not substitute other manufacturer's reagents. Dilution, reducing amplification reaction volumes or other deviation in this protocol may affect the performance of this test and/or nullify any limited sublicense that comes with the purchase of this testing kit.
- Materials are stable until the labeled expiration date when stored and handled as directed. Do not use kits beyond their expiration date.
- Adherence to the protocol will assure optimal performance and reproducibility. Ensure use of correct thermal cycler program is used, as other programs may provide inaccurate/faulty data, such as false positive and false negative results.
- Do not mix or combine reagents from kits with different lot numbers.
- Wear appropriate personal protective equipment and follow good laboratory practices and universal precautions when working with specimens. Handle specimens in approved biological safety containment facilities and open only in certified biological safety cabinets.
- Due to the analytical sensitivity of this test, use extreme care to avoid the contamination of reagents or amplification mixtures with samples, controls or amplified materials. Closely monitor all reagents for signs of contamination (*e.g.,* negative controls giving positive signals). Discard reagents suspected of contamination.
- To minimize contamination, wear clean gloves when handling samples and reagents and routinely clean work areas and pipettes prior to doing PCR. Autoclaving does not eliminate DNA contamination
- Follow uni-directional workflow between separate areas in the PCR laboratory: begin with master mix preparation, move to specimen preparation, then to amplification and finally to detection. Do not bring amplified DNA into the areas designated for master mix or specimen preparation.
- Dedicate all pipettes, pipette tips and any equipment used in a particular area to that area of the laboratory.
- Use sterile, disposable plastic ware whenever possible to avoid RNase, DNase or cross-contamination.

4.3. Storage and Handling

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

5. Instruments

5.1. Thermal cycler

- Use or function: Amplification of DNA samples
- Suggested instrument: Veriti™ thermal cycler or equivalent
- Performance characteristics and specification:
    - Minimum thermal range: 15°C to 96°C
    - Minimum ramping speed: 0.8°C/sec
- Follow manufacturer's installation, operation, calibration and maintenance procedures.
- See section 7.4 *Amplification* for thermal cycler program.

5.2. Electrophoresis Unit

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

5.3. UV Illumination Unit (for Gel Detection)

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

5.4. ABI Capillary Electrophoresis Instruments (for ABI Detection)

- Use or function: Fragment detection and analysis
- Performance characteristics and specification:
    - The following capillary electrophoresis instruments will meet the performance needs for this assay:
        - ABI 310 Genetic Analyzer (1-capillary)
        - ABI 3100 Avant Genetic Analyzer (4-capillaries)
        - ABI 3100 Genetic Analyzer (16-capillaries)
        - ABI 3130 Genetic Analyzer (4-capillaries)
        - ABI 3130xL Genetic Analyzer (16-capillaries)
        - ABI 3500 Genetic Analyzer (8-capillaries)
        - ABI 3500xL Genetic Analyzer (24-capillaries)
- Follow manufacturer's installation, operation, calibration and maintenance procedures.
- The ABI instrument used must be calibrated with appropriate Matrix Standards as outlined in section 7.2 *Materials Required (not provided).*
- Use the default settings for your polymer and capillary type, see section *7.5 Detection*.

6. Specimen Collection and Preparation

6.1. Precautions

Biological specimens from humans may contain potentially infectious materials. Handle all specimens in accordance with the OSHA Standard on Bloodborne Pathogens or Biosafety Level 2.

6.2. Interfering Substances

The following substances are known to interfere with PCR:

- Divalent cation chelators
- Low retention pipette tips
- EDTA (not significant at low concentrations)
- Heparin

6.3. Specimen Requirements and Handling

This assay tests extracted and purified genomic DNA (gDNA). Common sources of gDNA include:

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

6.4. Sample Preparation

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

6.5. Sample Storage

Store gDNA at 2°C to 8°C or at -85°C to -65°C for any duration other than immediate use.

7. Assay Procedure

7.1. Materials Provided

Table 3. Materials Provided

Gel Detection Kits	
Catalog #	Description
REF 22050010	*TCRB* Tube A – Unlabeled
REF 22050020	*TCRB* Tube B – Unlabeled
REF 22050030	*TCRB* Tube C – Unlabeled
REF 20960020	Specimen Control Size Ladder – Unlabeled
REF 40880490	IVS-0009 Clonal Control DNA
REF 40881210	IVS-0021 Clonal Control DNA
REF 40880190	IVS-0004 Clonal Control DNA
REF 40920010	IVS-0000 Polyclonal Control DNA

Differential Fluorescence Detection Kits	
Catalog #	Description
REF 22050011	*TCRB* Tube A – 6FAM & HEX
REF 22050021	*TCRB* Tube B – 6FAM
REF 22050031	*TCRB* Tube C – 6FAM & HEX
REF 20960021	Specimen Control Size Ladder – 6FAM
REF 40881210	IVS-0021 Clonal Control DNA
REF 40880490	IVS-0009 Clonal Control DNA
REF 40880190	IVS-0004 Clonal Control DNA
REF 40920010	IVS-0000 Polyclonal Control DNA

7.2. Materials Required (not provided)

Table 4. Materials Required (not provided)

Reagent/Material	Recommended Reagents/Materials and Suppliers	Part Number	Notes
DNA Polymerase	Roche: • EagleTaq DNA Polymerase Invivoscribe, Inc. • FalconTaq DNA Polymerase or equivalent	05206944190 60970130	N/A
Molecular Biology Grade or USP Water	N/A	N/A	DNase / RNase free
Calibrated Pipettes	Rainin: • P-2, P-20, P-200 and P-1000 pipettes or • SL-2, SL-20, SL-200 and SL-1000 pipettes	N/A	Capable of accurately measuring volumes between 1 µL and 1000 µL.
Thermal cycler	Thermo Fisher Scientific: • Veriti Thermal Cycler Bio-Rad: • MJ Research PTC-100 or PTC-200, PTC-220, PTC-240 Perkin-Elmer • PE 9600 or PE 9700	N/A	N/A
Vortex Mixer	N/A	N/A	N/A
PCR plates or tubes	N/A	N/A	Sterile
Filter barrier pipette tips	N/A	N/A	Sterile, RNase/DNase/Pyrogen-free
Microcentrifuge tubes	N/A	N/A	Sterile
Gel Detection			
Gel Electrophoresis Unit	N/A	N/A	For polyacrylamide gels
Ethidium Bromide	Thermo Fisher Scientific: • UltraPure™ 10 mg/mL Ethidium Bromide	15585-011	N/A
6% Polyacrylamide Gels	Thermo Fisher Scientific: • Novex® TBE Gels (6%, 12 well)	EC62652Box	N/A
TBE Running Buffer	Thermo Fisher Scientific: • Novex® TBE Running Buffer (5X)	LC6675	Dilute 1:5 prior to use.
Gel Loading Buffer	Thermo Fisher Scientific: • 10X BlueJuice™ Gel Loading Buffer • Novex® Hi-Density TBE Sample Buffer (5X)	10816-015 LC6678	N/A

Table 4. Materials Required (not provided)

Reagent/Material	Recommended Reagents/Materials and Suppliers	Part Number	Notes
100 bp DNA Ladder	Thermo Fisher Scientific: • TrackIt™ 100 bp DNA Ladder	10488-058	N/A
Ethidium Bromide	Thermo Fisher Scientific: • UltraPure™ 10 mg/mL Ethidium Bromide	15585-011	N/A
ABI Fluorescence Detection			
ABI Capillary Electrophoresis Instrument	Thermo Fisher Scientific: • ABI 310, 3100, or 3500 series	N/A	N/A
Hi-Di Formamide	Thermo Fisher Scientific: • Hi-Di™ Formamide	4311320	N/A

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

Spectral Calibration Dye Sets	Thermo Fisher Scientific:
	• For ABI 3100 instruments:
	  ○ DS-30 Matrix Standard Kit (Dye Set D)
	• For ABI 3500 instruments:
	  ○ DS-33 Matrix Standard Kit (Dye Set G5)
		4345827
		
		4345833
			N/A

Polymer	Thermo Fisher Scientific:
	• POP-4™ Polymer:
	  ○ POP-4 for 3100/3100-Avant Genetic Analyzers
	  ○ POP-4 for 3130/3130xL Genetic Analyzers
	• POP-7™ Polymer:
	  ○ POP-7 for 3130/3130xL Genetic Analyzers
	  ○ POP-7 for 3500/3500xL Genetic Analyzers
		
		4316355
		4352755
		
		4352759
		4393714
			N/A

Buffer	Thermo Fisher Scientific: • 10X Genetic Analyzer Buffer with EDTA	402824	Dilute 1:10 in sterile water before use

7.3. Reagent Preparation

- Unknown samples can be tested using the template amplification control (*e.g.,* **Specimen Control Size Ladder**) master mix to ensure that no inhibitors of amplification are present and there is sufficient DNA quality and quantity to generate a valid result.
- Test all samples in **singlicate**.
- Test **positive**, **negative** and **no template** controls for each of the master mixes.

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 a containment hood or dead air box, remove an appropriate aliquot of each master mix to individual, clean, sterile microfuge tube (one tube for each of the master mixes).

- Aliquot volumes are 45 µL per reaction, and we recommend adding an additional 20 µL to correct for pipetting errors.
- 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 (See Table 6)
	+ 1	negative control DNA (IVS-0000 Polyclonal Control DNA)
	+ 1	no template control (water)
	+ 1	to correct for pipetting errors
n =	# of samples + 4	Total

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

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. **For *TCRB* Tubes A and B:** Add 2.25 U (or 0.45 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 × 0.45 µL**.
- Gently vortex to mix.

7.3.4. **For *TCRB* Tube C and Specimen Control Size Ladder:** Add 1.25 U (or 0.25 µL @5 U/µL) of Taq DNA polymerase per reaction.

- The total DNA polymerase added to each master mix is **n × 0.25 µL** (*TCRB* Tube C master mix) or **m × 0.25 µL** (Specimen Control Size Ladder master mix).
- Gently vortex to mix.

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

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

7.3.7. Cap or cover the PCR plate.

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

7.3.8. 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.

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-100, PTC-200, or the PE 2600, 9600, or 9700 thermal cyclers.

Table 5. Thermal cycling conditions

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

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

- Although amplified DNA is stable at room temperature for extended periods of time, store PCR products at 2°C to 8°C until detection.

7.5. Detection

Gel Detection – Agarose TBE Gels

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

7.5.2. Mix 20 µL from each amplification reaction and individually mix with 4 µL of 6X gel loading buffer.

- Load 20 µL of this mixture into separate wells of the gel, flanking with the DNA size standards.
- Detect the amplified products with ethidium bromide or an equivalent dye.

7.5.3. Photograph the gel and interpret the data.

Gel Detection – Polyacrylamide TBE Gels

7.5.4. Assemble electrophoresis unit using a 6% non-denaturing polyacrylamide TBE gel and 0.5X TBE running buffer.

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

7.5.6. Load 20 µL of mixture into wells of the gel.

7.5.7. Run gel at 110V for 2-3 hours or 40-50V overnight.

- Voltage and electrophoresis time depend on the PCR amplicon size, acrylamide gel thickness and type of PCR equipment.
- Voltage and run time can be adapted accordingly.

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

7.5.9. De-stain gels 2X in water for 5-10 minutes.

7.5.10. Using UV illumination, visualize and photograph the gel then interpret the data.

Gel Detection – Heteroduplex Analysis (RECOMMENDED)

7.5.11. Denature 20 µL PCR products at 94ºC for 5 minutes.

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

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

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

7.5.15. Load 20 µL of mixture into wells of the gel.

7.5.16. Run gel at 110V for 2-3 hours or 40-50V overnight.

- Voltage and electrophoresis time depend on the PCR amplicon size, acrylamide gel thickness and type of PCR equipment.
- Voltage and run time can be adapted accordingly.

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

7.5.18. De-stain gels 2X in water for 5-10 minutes.

7.5.19. Using UV illumination, visualize and photograph the gel then interpret the data.

ABI Fluorescence Detection with ABI 310, 3100 & 3130 instruments

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

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

7.5.22. 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.

7.5.23. Cap or cover the PCR plate.

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

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

7.5.26. Run the samples on an ABI 310/3100/3130 capillary electrophoresis instrument following the user manual.

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

7.5.27. Review the profile and controls, and report the results.

ABI Fluorescence Detection with ABI 3500 instruments

Note: Due to instrument to instrument variation in the performance of the ABI 3500 platform, the amount of formamide, sample and size standard listed in the protocol is intended to be a starting point. The protocol may need to be optimized for specific ABI 3500 Platforms.

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

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

7.5.30. Transfer 0.5 µL of each reaction to the wells containing Hi-Di Formamide with LIZ size standards.

- Add only one sample per well.
- Pipette up and down to mix.

7.5.31. Cap or cover the PCR plate.

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

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

7.5.34. Run the samples on an ABI 3500 capillary electrophoresis instrument following the user manual.

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

7.5.35. Review the profile and controls, and report the results.

[a]Note: Please see Applied Biosystems' accompanying product insert for mixing Hi-Di Formamide with size standards for different ABI instruments.

7.6. Template Amplification Controls

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

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

7.7. Recommended Positive Controls

The amplicon sizes listed were determined using an ABI platform. Amplicon sizes seen on each specific capillary electrophoresis instrument may differ 1 to 4 bp from those listed depending on the platform of detection and the version of the analysis software used. Once identified, the amplicon size as determined on each specific platform will be consistent from run to run. 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. Recommended Positive Controls

Master Mix	Target	Color	Control DNA	Catalog #	Product Size (bp)
TCRB Tube A	Vβ + Jβ1/2	Blue (Jβ2.X) + Green (Jβ1.X)	**Valid Size Range** IVS-0009 Clonal Control DNA	--- 40880490	**240-285** 264
TCRB Tube B	Vβ + Jβ2	Blue (Jβ2.X)	**Valid Size Range** IVS-0004 Clonal Control DNA IVS-0021 Clonal Control DNA	--- 40880190 40881210	**240-285** 253 267
TCRB Tube C	Dβ + Jβ1/2	Blue (Jβ2.X) + Green (Jβ1.X)	**Valid Size Range** IVS-0009 Clonal Control DNA	--- 40880490	**170-210 (Dβ2), 285-325 (Dβ1)** 309
Specimen Control Size Ladder	Multiple Genes	Blue	**Valid Size Range** IVS-0000 Polyclonal Control DNA	--- 40920010	**96, 197, 297, 397, 602[a]** 96, 197, 297, 397, 602[a]

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

8. Interpretation and Reporting

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 Next Gen Sequencing (NGS) may be advisable to rule out clonality.

8.1. Analysis

The size range for each master mix has been determined testing positive control samples. For accurate and meaningful interpretation, ignore peaks that occur outside of the valid size range for each master mix. Peaks that are outside of the range cannot be assumed to be valid.

Results can be reported as "**Positive**" or "**Negative**" for "**Detection of clonal T-cell receptor beta chain gene rearrangement, consistent with the presence or absence of a clonal cell population**"

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

8.1.2. All assay controls must be examined prior to interpretation of sample results.

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

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

8.2. Sample Interpretation

Following the acceptance of the controls, the samples are interpreted as follows: One or two prominent bands within the valid size range are reported as "**Detection of clonal T-cell receptor beta chain gene rearrangement is consistent with the presence of a clonal cell population**."

9. Limitations of Procedure

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

10. Expected Values

10.1. Expected Size of Amplified Products

Note: "Color" indicates the color of products generated with the master mix when using the default color assignment on ABI fluorescence detection systems. The amplicon sizes listed were determined using an ABI platform.

Table 7. Expected Size of Amplified Products

Master Mix	Mix	Color	Control DNA	Catalog #	Product Size (bp)
TCRB Tube A	Vβ + Jβ	Blue (J β2.X) + Green (J β1.X)	**Valid Size Range** IVS-0000 Polyclonal Control DNA IVS-0009 Clonal Control DNA IVS-0004 Clonal Control DNA	--- 40920010 40880490 40880190	**240 - 285** 240 - 285, 270[1] 264 295
TCRB Tube B	Vβ + Jβ2	Blue (J β2.X)	**Valid Size Range** IVS-0000 Polyclonal Control DNA IVS-0009 Clonal Control DNA IVS-0004 Clonal Control DNA IVS-0021 Clonal Control DNA	--- 40920010 40880490 40880190 40881210	**240 - 285** 240 - 285, 221[2] --- 253 267
TCRB Tube C	Dβ + Jβ	Blue (Jβ2.X) + Green (J β1.X)	**Valid Size Range** IVS-0000 Polyclonal Control DNA IVS-0009 Clonal Control DNA IVS-0004 Clonal Control DNA	--- 40920010 40880490 40880190	**170 - 210 (Dβ2), 285 - 325 (Dβ1)** 128[2], 170 - 210, 285 - 325, 337[2] 309 295
Specimen Control Size Ladder	Multiple Genes	Blue	Any Human DNA	---	**96, 197, 297, 397, 602**[3] 96, 197, 297, 397, 602[3]

Note: The amplicon sizes listed above were determined using an ABI platform. Amplicon sizes seen on each CE instrument may vary 1-4 bp from those listed above depending on the platform of detection (ABI) and the version of the analysis software used. Once identified, the amplicon size as determined on your specific platform will be consistent from run to run.

[1]**Note:** The 273 bp band (mainly visible with GeneScan analysis) is particularly seen in samples with low numbers of contaminating lymphoid cells.

[2]**Note:** Under sub-optimal conditions, nonspecific products of 128, 221, and 337 bp can be detected in Tubes B and C. If present, these bands will normally be faint.

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

10.2. Sample Data

Gel Detection

The data shown in Figure 2 - Figure 5 were generated using the master mixes indicated and run on a 6% polyacrylamide gel.

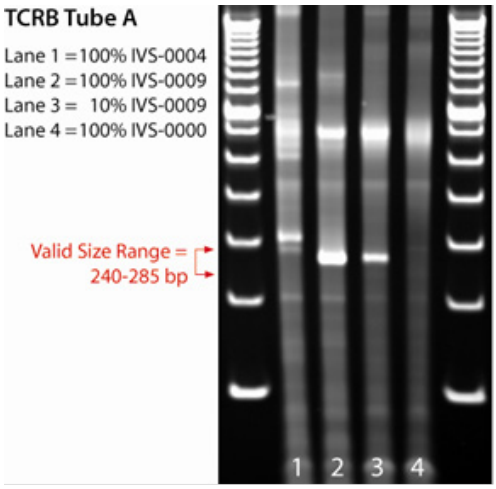


Figure 2. *TCRB* Tube A – Unlabeled master mix.

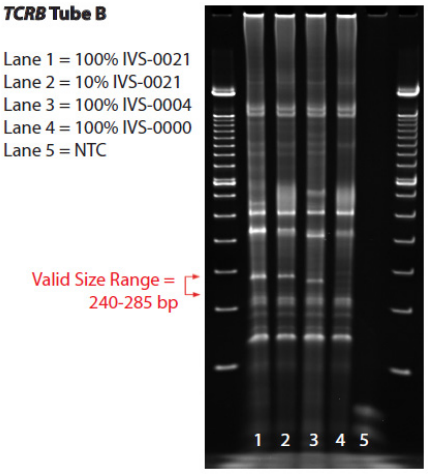


Figure 3. *TCRB* Tube B - Unlabeled master mix.

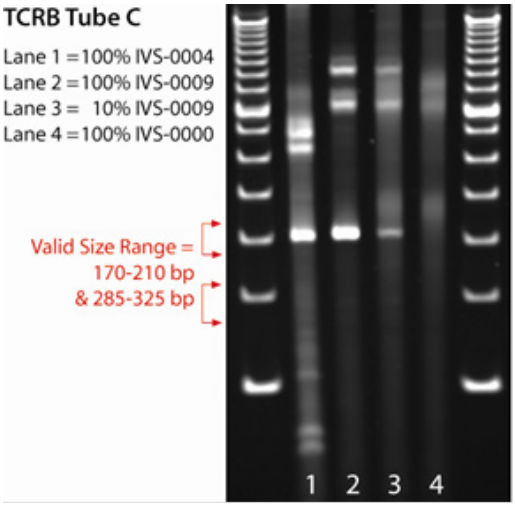


Figure 4. *TCRB* Tube C - Unlabeled master mix.

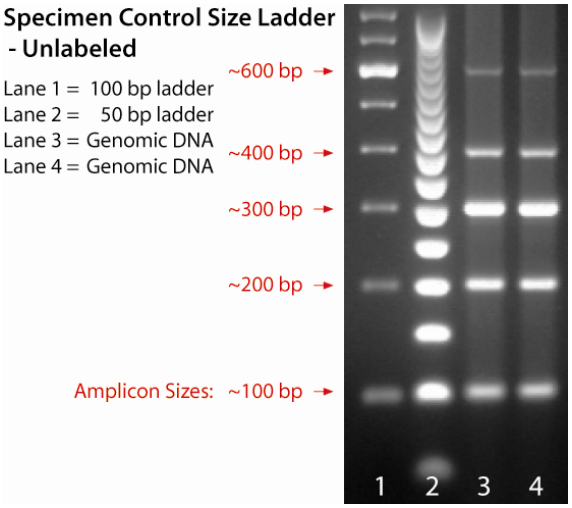


Figure 5. Amplified products were generated with the Specimen Control Size Ladder - Unlabeled master mix and run on a 2% agarose gel.

ABI Fluorescence Detection

The data shown in Figure 6 - Figure 9 were generated using the master mixes indicated. Amplified products were run on an ABI instrument

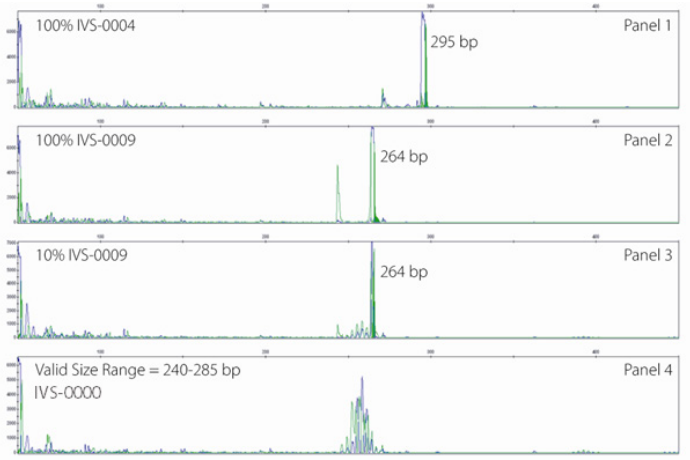


Figure 6. *TCRB* Tube A – 6FAM & HEX master mix.

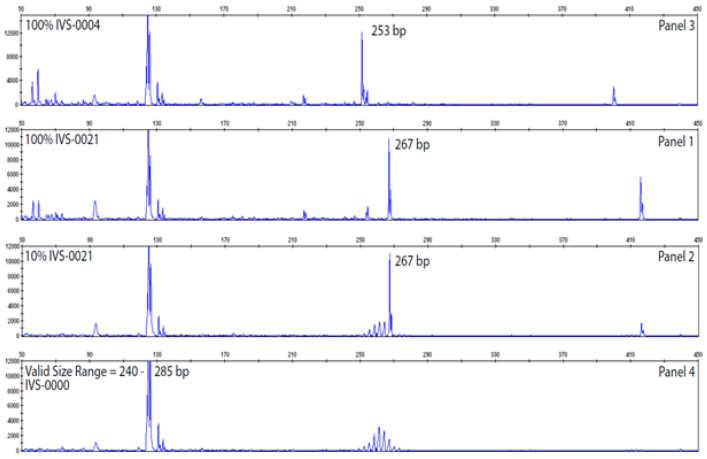


Figure 7. *TCRB* Tube B – 6FAM master mix.

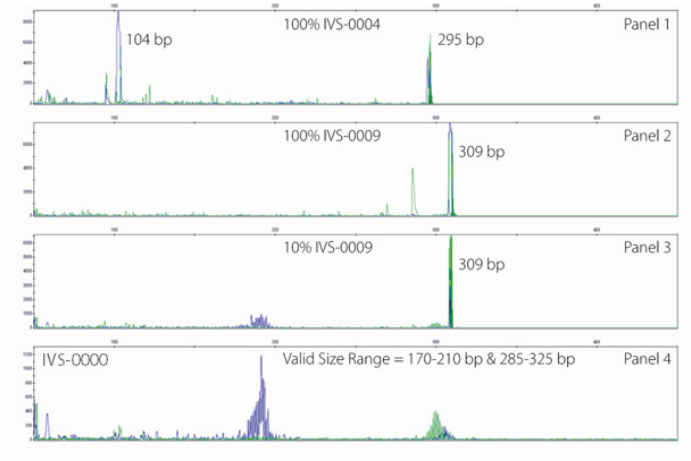


Figure 8. *TCRB* Tube C – 6FAM & HEX master mix.

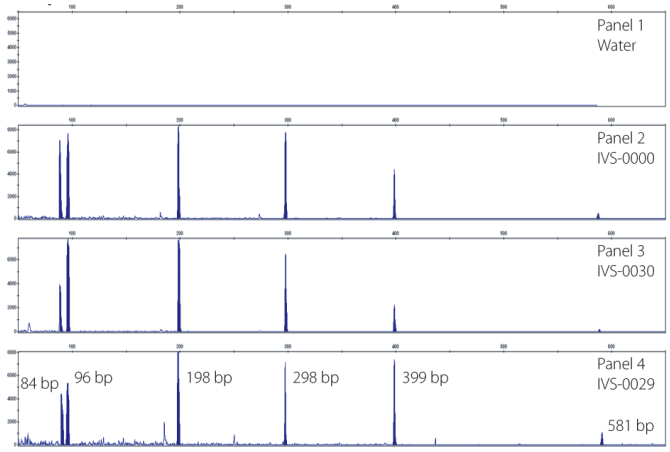


Figure 9. Specimen Control Size Ladder – 6FAM master mix

11. Technical and Customer Service

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

Contact Information

Invivoscribe, Inc

10222 Barnes Canyon Road | Building 1 | San Diego | California 92121-2711 | USA

Phone: +1 858 224-6600 | Fax: +1 858 224-6601 | Business Hours: 7:00AM - 5:00 PM PST/PDT

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

12. References

1. Miller, JE, Wilson, SS, Jaye, DJ, Kronenberg, M. An automated semiquantitative B and T cell clonality assay. *Mol. Diag*. 1999, **4(2)**:101-117.
2. van Dongen, JJM *et al*. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: Report of the BIOMED-2 Concerted Action BMH4-CT98-3936. *Leukemia*. 2003, **17(12)**:2257-2317.
3. van Krieken JH, Langerak AW, Macintyre EA, Kneba M, Hodges E, Sanz RG, Morgan GJ, Parreira A, Molina TJ, Cabeçadas J, Gaulard P, Jasani B, Garcia JF, Ott M, Hannsmann ML, Berger F, Hummel M, Davi F, Brüggemann M, Lavender FL, Schuuring E, Evans PA, White H, Salles G, Groenen PJ, Gameiro P, Pott Ch, van Dongen JJM. Improved reliability of lymphoma diagnostics via PCR-based clonality testing: report of the BIOMED-2 Concerted Action BHM4-CT98-3936. *Leukemia*. 2007; **21(2)**:201-6.

13. Symbols

The following symbols are used in labeling for Invivoscribe products.

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

14. Legal Notice

14.1. Warranty and Liability

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

This product is for Research Use Only.

14.2. Patents and Trademarks

This product is covered by one or more of the following: European Patent Number 1549764, European Patent Number 2418287, European Patent Number 2460889, Japanese Patent Number 4708029, United States Patent 8859748, and related pending and future applications. All of these patents and applications are licensed exclusively to Invivoscribe®. Additional patents licensed to Invivoscribe covering some of these products apply elsewhere. Many of these products require nucleic acid amplification methods such as Polymerase Chain Reaction (PCR). No license under these patents to use amplification processes or enzymes is conveyed expressly or by implication to the purchaser by the purchase of this product.

15. *TCRB* Gene Clonality Assay: Single Page Guide

1. Using gloved hands, remove the master mixes from the freezer. Allow the tubes to thaw; then gently vortex to mix.
2. In a containment hood or dead air box remove an appropriate aliquot to clean, sterile microfuge tube (one tube for each master mix).
   - **Aliquot volumes = 45 µL** for each reaction (n = # reactions, plus positive control, negative control and NTC).
   - **Total volume = 45 µL × (n + 4)**
3. Add the appropriate amount of Taq DNA polymerase to each master mix and gently mix by inverting several times or gently vortexing.
   - **Tubes A and B:** 0.45 µL of Taq (@5 U/µL) per 50 µL total reaction volume
   - **Tube C:** 0.25 µL of Taq (@5 U/µL) per 50 µL total reaction volume
4. Aliquot 45 µL of master mix to individual wells of a PCR plate.
5. Add 5 µL of DNA from the unknown and control samples to individual tubes or wells containing the respective master mix reactions and pipette up and down several times to mix.
6. Amplify target DNA using the universal thermal cycler program.

Gel Detection – Heteroduplex Analysis

7. Denature 20 µL of PCR products at 94ºC for 5 minutes.
8. Re-anneal PCR products at 4ºC for 60 minutes.
9. Assemble electrophoresis unit using a 6% non-denaturing polyacrylamide TBE gel and 0.5X TBE running buffer.
10. Add 5 µL ice-cold non-denaturing bromophenol blue loading buffer to samples
11. Load 20 µL of mixture into wells of the gel.
12. Run gel at 110V for 2-3 hours or 40-50V overnight.
13. Stain gels in 0.5 µg/mL ethidium bromide (in water or 0.5X TBE Buffer) for 5-10 minutes.
14. De-stain gels 2X in water for 5-10 minutes.
15. Using UV illumination, visualize and photograph the gel then interpret the data.

ABI Fluorescence Detection with ABI 310, 3100 & 3130 instruments

7. In a new microcentrifuge tube, mix an appropriate amount (10 µL per reaction) of Hi-Di Formamide with ROX Size Standards. Vortex well.
8. In a new 96-well PCR plate, add 10 µL of Hi-Di Formamide with ROX size standards to individual wells for each reaction.
9. 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.
10. Cap or cover the PCR plate.
11. Heat denature the samples at 95ºC for 2 minutes, then snap chill on ice for 5 minutes.
12. Prepare a sample sheet and injection list for the samples.
13. Run the samples on an ABI 310/3100/3130 capillary electrophoresis instrument following the user manual.
14. Review the profile and controls, and report the results.

ABI Fluorescence Detection with ABI 3500 instruments

Note: Due to instrument to instrument variation in the performance of the ABI 3500 platform, the amount of formamide, sample and size standard listed in the protocol is intended to be a starting point. The protocol may need to be optimized for specific ABI 3500 Platforms.

7. In a new microcentrifuge tube, mix an appropriate amount (9.5 µL per reaction) of Hi-Di Formamide with LIZ Size Standards. Vortex well.
8. In a new 96-well PCR plate, add 9.5 µL of Hi-Di Formamide with LIZ size standards to individual wells for each reaction.
9. Transfer 0.5 µL of each reaction to the wells containing Hi-Di Formamide with LIZ size standards. Add only one sample per well. Pipette up and down to mix.
10. Cap or cover the PCR plate.
11. Heat denature the samples at 95ºC for 3 minutes, then snap chill on ice for 5 minutes.
12. Prepare a sample sheet and injection list for the samples.
13. Run the samples on an ABI 3500 capillary electrophoresis instrument following the user manual.
14. Review profile and controls, report results.