Instructions for Use

BCL2/JH Translocation Assay

For Identification of Follicular Cell and other Lymphomas and Leukemias

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

t(14;18) tube A: 2 *BCL2* MBR primers + 1 JH primer

t(14;18) tube B: 4 *BCL2* 3'MBR primers + 1 JH primer

t(14;18) tube C: 3 *BCL2* mcr primers + 1 JH primer

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Manufactured in U.S.A.

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

Catalog#	Products	Quantity
REF 1-309-0020	*BCL2*/JH Translocation Assay for Gel Detection	33 reactions
REF 1-309-0040	*BCL2*/JH Translocation Assay MegaKit for Gel Detection	330 reactions

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Thank you for purchasing our ***BCL2*/JH Translocation 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.** Questions received during business hours usually receive a response within an hour. Alternatively, you can call for technical assistance and for information on our testing kits at (858) 224-6600 between the hours of 8:00 AM and 5:00 PM Pacific Standard Time.

1. Notice

This product is covered by one or more of the following patents and patent applications owned by or exclusively licensed to Invivoscribe Technologies, Inc. (IVS): United States Patent Application Number 10/531,106, European Patent Number EP 1549764B1 and other pending patent applications originating from European Patent Application Numbers 03756746.8 (16 countries) and Japanese Patent Number JP04708029B2.

Use of this product may require nucleic acid amplification methods such as Polymerase Chain Reaction (PCR). Any necessary license to practice amplification methods or to use amplification enzymes or equipment covered by third party patents is the responsibility of the user and no such license is granted by Invivoscribe Technologies, Inc., expressly or by implication. This product is sold **FOR RESEARCH USE ONLY; not for use in diagnostic procedures**.

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

2. Principle

NOTICE: Invivoscribe Technologies' Gene Rearrangement and Translocation Assays represent a new approach to PCR-based clonality testing. These standardized assays were carefully optimized testing positive and negative control samples using multiplex master mixes. Assay development was followed by extensive validation testing more than 400 clinical samples using Revised European/American Lymphoma (REAL) Classification. Testing was done at more than thirty prominent independent testing centers throughout Europe in a collaborative study known as the BIOMED-2 Concerted Action. Results from this BIOMED-2 study appear in a leading peer-reviewed journal, Leukemia. 2003 Dec;17(12): 2257-2317 (Nature Publishing Group).

BACKGROUND:

BCL2 translocations are reciprocal chromosome exchanges that place the *bcl*-2 proto-oncogene, located on chromosome 18, under aberrant transcriptional control of the immunoglobulin heavy chain gene, located on chromosome 14. The bcl-2 protein is an antagonist to apoptosis (programmed cell death), a normal process designed to eliminate unneeded and damaged cells during hematopoiesis. Increased expression of the bcl-2 protein leads to an increase in the levels of B cells in the body. *BCL2* t(14;18) translocations are present in 70-90% of follicular non-Hodgkin B cell lymphomas, 50% of undifferentiated B cell lymphomas and 20-30% of large cell diffuse B cell lymphomas. They are not seen in other lymphomas; therefore, this test is useful for the differential diagnosis of B cell malignancies. Further, presence of the bcl-2 translocation is an indicator of poor prognosis in large cell diffuse B cell lymphomas.

This *BCL2* t(14;18) Translocation Assay contains four master mixes. Three of the master mixes target the joining region of the immunoglobulin heavy chain gene and distinct regions of the *BCL2* gene. These master mixes are used to detect major breakpoint region (Mbr) and minor cluster region (mcr) of the *BCL2* t(14;18) translocations.

The fourth master mix, the Specimen Control Size Ladder, targets multiple genes and generates a series of amplicons of 100, 200, 300, 400 and 600 base pairs to ensure that the quality and quantity of input DNA is adequate to yield a valid result. Positive and negative control DNAs and also included. This assay is used both to identify and monitor *BCL2* translocations involving the major breakpoint region (Mbr) and the minor cluster region (mcr). The assay can also be used to detect disease recurrence.

3. 3. Assay Uses

***BCL2*/JH Translocation Assays are useful for the study of:**

- Monitoring and evaluation of follicular lymphomas and other B cell lymphomas
- Distinguishing lymphoma from benign lymphoid hyperplasia
- Distinguishing follicular lymphoma from other B cell lymphomas that may have a similar appearance
- Monitoring and evaluation of disease recurrence
- Detection and assessment of residual disease
- Evaluation of new research and methods in malignancy studies

4. Specimen Requirements

- **This assay tests genomic DNA**

1. 5 cc of peripheral blood, bone marrow biopsy or bone marrow aspirate anti-coagulated with heparin or EDTA. Ship at ambient temperature; OR
2. Minimum 5 mm cube of tissue shipped frozen; or at room temperature or on ice in RPMI 1640; OR
3. 2 µg of genomic DNA; OR
4. Formalin-fixed paraffin embedded tissue or slides.

5. Kit Contents

Controls and Standards	IVS Catalog #	Concentration
IVS-0030 Clonal Control DNA	4-088-1750	100 µL @200 µg/mL
IVS-P002 Clonal Control DNA	4-090-0070	100 µL @200 µg/mL
IVS-0031 Clonal Control DNA	4-088-1810	100 µL @200 µg/mL
IVS-0000 Polyclonal Control DNA	4-092-0010	100 µL @200 µg/mL

Master Mixes	IVS Catalog #	Target
BCL2/JH Tube A	2-309-0050	*BCL2* MBR + IGH JH
BCL2/JH Tube B	2-309-0060	*BCL2* 3' MBR + IGH JH
BCL2/JH Tube C	2-309-0070	*BCL2* mcr + IGH JH
Specimen Control Size Ladder	2-096-0020	Multiple Genes

Note: MegaKits contain 10 units of each master mix and 5 units of each Controls and Standards

STATEMENT OF WARNINGS

The assay kit has been optimized to 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. Close adherence to the protocol will assure optimal performance and reproducibility. It is recommended that glass distilled de-ionized molecular biology grade water be used with the preparation of specimen DNA. This can be purchased from several manufacturers. In addition, 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. Please see Section 9 for further details.

6. Storage Conditions

PCR master mixes are sensitive to freeze/thaw cycles. Therefore, for any duration other than immediate use, our **master mixes and assay kits should be stored at -65°C to -85°C**.

The reason for this is quite straightforward: Due to the high salt concentrations in our master mixes, the effective freezing and thawing temperature of the master mixes is approximately –10°C. The temperature in a standard laboratory –20°C freezer can easily reach –10°C or warmer during the day when these freezers are opened on a regular basis. At these temperatures, PCR master mixes may go through multiple freeze/thaw cycles, resulting in precipitation of the primers. Accordingly, to minimize the exposure of your master mixes to freeze/thaw cycles, **IVS recommends that master mixes be stored at -65°C to -85°C**.

Please note that our DNA standards are best stored at 2°C to 8°C. However, these standards can be stored at any lower temperature as long as they are vortexed after thawing and before use to ensure that they are re-suspended completely

If you have any questions, please contact our technical staff. We are happy to help you determine your optimal storage needs.

7. Reagents Required But Not Included

PCR Amplification

AmpliTaq Gold DNA Polymerase or equivalent **(RECOMMENDED)**	(Life Technologies, Cat# N808-0241)
EagleTaq DNA Polymerase or equivalent **(RECOMMENDED)**	(Roche Cat# 05206944190)
AmpliTaq DNA Polymerase	(Life Technologies, Cat# N808-0161)

8. Recommended Positive Controls

Master Mix	Target	Control DNA	Cat#	Product Size in Base pairs
Tube A	*BCL2* Mbr + *IGH* JH	**Valid Size Range** IVS-0030 Clonal Control DNA	--- 4-088-1750	**100-2500** ~250, ~750[1]
Tube B	*BCL2* 3' Mbr + *IGH* JH	**Valid Size Range** IVS-P002 Clonal Control DNA	--- 4-090-0070	**100-2500** 138
Tube C	*BCL2* mcr + *IGH* JH	**Valid Size Range** IVS-0031 Clonal Control DNA	--- 4-088-1810	**100-2500** ~390, ~800
Specimen Control Size Ladder	Multiple Genes	**Valid Size Range** IVS-0000 Polyclonal Control DNA	--- 4-092-0010	**84, 96, 200, 300, 400, 600** 84, 96, 200, 300, 400, 600

9. Procedure Notes

- 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.

1. Do not bring amplified DNA into the areas designated for master mix or specimen preparation.
2. 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.
3. All pipettes, pipet tips and any equipment used in a particular area should be dedicated to and kept to that area of the laboratory.

4. PCR trays, bases and retainers must to be decontaminated in 10% bleach and rinsed with distilled water two separate times before returning them to the starting areas.
5. Sterile, disposable plastic ware should be used whenever possible to avoid RNase or cross-contamination.

10. 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 DNA of sufficient quality and quantity to generate a valid result.
- All samples should be tested in **singlicate**.
- **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; then gently vortex to mix.
2. In containment hood or dead air box remove an appropriate aliquot to clean, sterile microfuge tube (one tube for each of the master mixes). Aliquot volumes should be 45 µL for each sample + 135 µL (3 x 45 µL) for the positive, negative and no template controls. We recommend adding an additional 20 µL to correct for pipetting errors.
3. Add the appropriate amount of either AmpliTaq Gold, EagleTaq or AmpliTaq DNA polymerase (0.25 µL of either AmpliTaq Gold, EagleTaq or AmpliTaq @5 U/µL per 50 µL total reaction volume) to each of the master mixes and gently mix by inverting several times or gently vortexing.

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

11. Sample Preparation

Using any method of DNA extraction, extract the genomic DNA from unknown samples. Resuspend DNA to final concentration of 100 µg – 400 µg per mL in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) or distilled water. This is a robust assay system. A wide range of DNA concentrations will generate a valid result. Therefore, quantifying and adjusting DNA concentrations is generally not necessary. Testing sample DNAs with the Specimen Control Size Ladder master mix will ensure that DNA of sufficient quality and quantity was present to yield a valid result.

12. Amplification

1. Aliquot 45 µL of the master mix/enzyme solutions into individual PCR wells or tubes.
2. Add 5 µL sample or control DNA to the individual tubes or wells containing the respective master mix reactions. Pipette up and down several times to mix. Amplify the reactions using the following PCR program.

We recommend the MJ Research PTC-100, PTC-200 or the PE 2600, 9600 or 9700 thermal cyclers, using the following PCR parameters for the amplifications:

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

(RECOMMENDED) **Standard Program for AmpliTaq Gold or EagleTaq**	Modified Program for AmpliTaq
Step 1: 95°C for 7 minutes	Step 1: 95°C for 7 minutes
Step 2: 95°C for 45 seconds	Step 2: 95°C for 45 seconds
Step 3: 60°C for 45 seconds	Step 3: 60°C for 45 seconds
Step 4: 72°C for **90 seconds**	Step 4: 72°C for **120 seconds**
Step 5: Go to step 2; 34 more times	Step 5: Go to step 2; 34 more times
Step 6: 72°C for 10 minutes	Step 6: 72°C for 10 minutes
Step 7: 15°C forever	Step 7: 15°C forever

Remove the amplification plate from the thermal cycler

13. Detection

- **Not all detection formats are available for all assays**

Available Template Amplification Controls

- The **Specimen Control Size Ladder** master mix produces amplicons at ~100, 200, 300, 400 and 600 base pairs. Please note that the ~100 bp band is comprised of 84 bp and 96 bp bands. Both of these bands co-migrate on a gel. The products of this master mix should be run separately.

Gel Detection – Agarose TBE Gels (RECOMMENDED)

1. A 2% MetaPhor or NuSieve 3:1 agarose/TBE gel is prepared.
2. 20 µL from each of the amplification reactions are individually mixed with 4 µL of 6X gel loading buffer. 20 µL of this mixture is loaded into separate wells of the gel, flanked by DNA size standards. Products are detected using ethidium bromide or an equivalent dye.
3. Gel is photographed and data are interpreted.

Gel Detection – Polyacrylamide TBE Gels

1. Assemble electrophoresis unit using a 6% non-denaturing polyacrylamide TBE gel (made with 1X TBE, Invitrogen Cat# EC62652Box) and 0.5X TBE running buffer (Invitrogen 5X TBE Cat# LC6675).
2. Add 5 µL of ice-cold non-denaturing bromophenol blue loading buffer to samples.
3. Load 20 µL of mixture into 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, acrylamide gel thickness and type of PCR equipment. Voltage and run time can be adapted accordingly.
5. Gels are stained in 0.5 µg/mL EtBr (in water or 0.5X TBE Buffer) for 5-10 minutes.
6. Gels are destained 2X in water for 5-10 minutes.
7. UV illumination is used for visualization.
8. Gel is photographed and data are interpreted.

Gel Detection – Heteroduplex Analysis

1. Denature 20 µL PCR products at 94ºC for 5 minutes.
2. Re-anneal PCR products at 4ºC for 60 minutes.
3. Assemble electrophoresis unit using a 6% non-denaturing polyacrylamide TBE gel (made with 1X TBE, Invitrogen Cat# EC62652Box) and 0.5X TBE running buffer (Invitrogen 5X TBE Cat# LC6675).
4. Add 5 µL ice-cold non-denaturing bromophenol blue loading buffer to samples
5. Load 20 µL of mixture into wells of the gel.
6. 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. Gels are stained in 0.5 µg/mL EtBr (in water or 0.5X TBE Buffer) for 5-10 minutes.
8. Gels are destained 2X in water for 5-10 minutes.
9. UV illumination is used for visualization.
10. Gel is photographed and data are interpreted.

14. Interpretation and Reporting

Note: This assay is for research use only. Although positive results are highly suggestive of malignancy, these assays are designed for Research Use Only and, if used in a clinical setting, should only be used in support of diagnosis. Positive and negative results should be interpreted in the context of all clinical information and laboratory test results. PCR based testing does not identify 100% of clonal cell populations; therefore, repeat testing by Southern blot may be advisable to rule out clonality.

The size range for each of the master mixes has been determined testing positive control samples. For accurate and meaningful interpretation it is important to ignore bands that occur outside of the proscribed/valid size range for each of the master mixes. Bands that are outside of the range cannot be assumed to be valid.

Note: The mix uses a consensus JH primer. Therefore, in a positive specimen, several valid products are often produced. These amplicons differ in size by about 500 bp; the distance between intron segments between these tandem JH exons.

Expected Size of Amplified Products

Master Mix	Target	Control DNA	Cat#	Product Size in base pairs
Tube A	*BCL2* Mbr + *IGH* JH	**Valid Size Range**	---	**100-2500**
		IVS-0000 Polyclonal Control DNA	4-092-0010	---
		IVS-0030 Clonal Control DNA	4-088-1750	~250, ~750[1]
		IVS-0031 Clonal Control DNA	4-088-1810	---
Tube B	*BCL2* 3' Mbr + *IGH* JH	**Valid Size Range**	---	**100-2500**
		IVS-0000 Polyclonal Control DNA	4-092-0010	---
		IVS-0030 Clonal Control DNA	4-088-1750	---
		IVS-P002 Clonal Control DNA	4-090-0070	138
		IVS-0031 Clonal Control DNA	4-088-1810	---
Tube C	*BCL2* mcr + *IGH* JH	**Valid Size Range**	---	**100-2500**
		IVS-0000 Polyclonal Control DNA	4-092-0010	---
		IVS-0030 Clonal Control DNA	4-088-1750	---
		IVS-0031 Clonal Control DNA	4-088-1810	~390, ~800
Specimen Control Size Ladder	Multiple Genes	Any Human DNA	---	84, 96, 200, 300, 400, 600

Note: The amplicon sizes listed above were determined using an ABI 3100 platform. **Note**[1]: This 750 bp band is generally seen as a very weak band.

Results can be reported as "**Positive**" or "**Negative**" for "**presence of the *BCL2*/JH t(14;18) Translocation**"

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. It is acceptable to call a sample "**Positive**" when a translocation product is generated yet the positive control for that master mix fails.
3. Samples that test negative should be repeated if the positive control reaction failed.
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.

- **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 assay unless specimen tests positive
- **Specimen Control Size Ladder**: (This is run on unknown samples only). If the amplification control is:
  - **Positive**: ~100, 200, 300, 400 and 600 base pair products are seen. Because smaller PCR fragments are preferentially amplified, it is not unusual for the 600 base pair fragment to have a diminished signal or to be missing entirely. Continue with analysis.
  - **Negative**: Repeat assay unless specimen tests positive.

Sample Interpretation

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

One or two prominent bands within the 100 - 2500 bp range are reported as**:**
"**Detection of a *BCL2*/JH t(14;18) Translocation**"

15. Limitations of Procedure

The assay is subject to interference by degradation of DNA or inhibition of PCR due to heparin or other agents. The assay cannot reliably detect less than 1 positive cell per 100 normal cells.

16. References

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.

17. Appendix

Reagents and Special Supplies

Ficoll Separation

Ficoll-Hypaque or Ficoll-Paque	(Pharmacia, Cat# 17-0840-02)
1X PBS diluted from 10X PBS	(Gibco/BRL, Cat# 70011-044)
RPMI 1640	(Gibco/BRL, Cat# 11875-093)
DMSO Hybri-Max	(Sigma, Cat# D2650)
Fetal Bovine Serum	(Hyclone, Cat# SH30071.03)

Gel Electrophoresis

MetaPhor Agarose, 125 g	(Cambrex,	Cat# 50180)
NuSieve 3:1 Agarose, 125 g	(Cambrex,	Cat# 50090)
UltraPure™ 10 mg/mL Ethidium Bromide	(Invitrogen,	Cat# 15585-011)
10X BlueJuice™ Gel Loading Buffer	(Invitrogen,	Cat# 10816-015)
Ready-Load™ 100 bp Ladder	(Invitrogen,	Cat# 10380-012)
Novex® TBE gels (6%, 12 well)	(Invitrogen,	Cat# EC62652Box)
Novex® TBE Running Buffer (5X)	(Invitrogen,	Cat# LC6675)
Novex® Hi-Density TBE Sample Buffer (5X)	(Invitrogen,	Cat# LC6678)

18. Troubleshooting Guide

Our laboratories are located in San Diego, California. Technical assistance is most rapidly obtained using our Internet site: **http://www.invivoscribe.com** or by sending an email inquiry to: **support@invivoscribe.com**. Alternatively, you can call (858) 224-6600 for technical assistance and information on our testing kits between the hours of 8:00 AM and 5:00 PM Pacific Standard Time.

Questions received during business hours usually receive a response within an hour.

19. Sample Data

Gel Detection

The data shown in Figures 1 - 3 were generated using the master mixes indicated.

- Lane 1 displays data generated testing an alternative 100% clonal control DNA.
- Lane 2 displays data generated testing the recommended 100% clonal control DNA.
- Lane 3 displays data generated testing a 1% dilution of the recommended clonal control DNA.
- Lane 4 displays data generated testing IVS-0000 Polyclonal Control DNA.

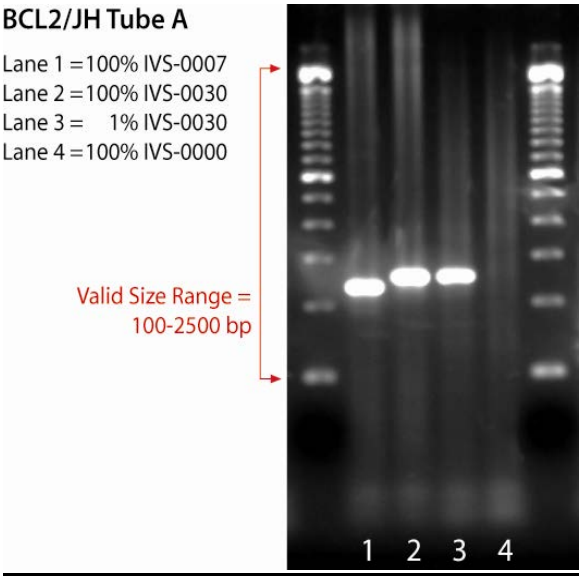

Figure 1. *BCL2*/JH Tube A master mix run on a 2% agarose gel.

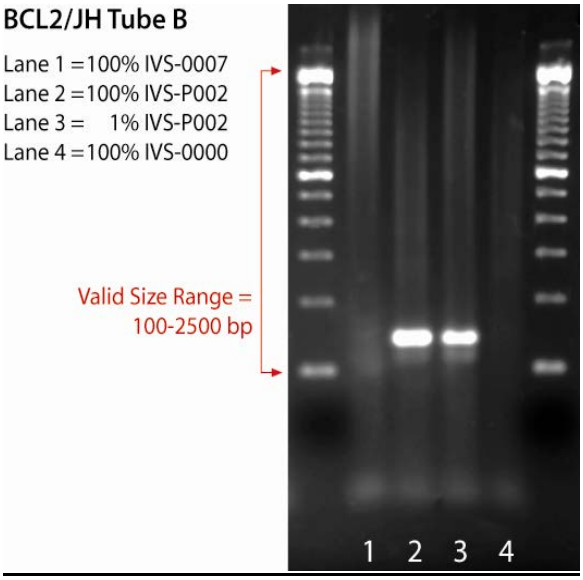

Figure 2. *BCL2*/JH Tube B master mix run on a 2% agarose gel.

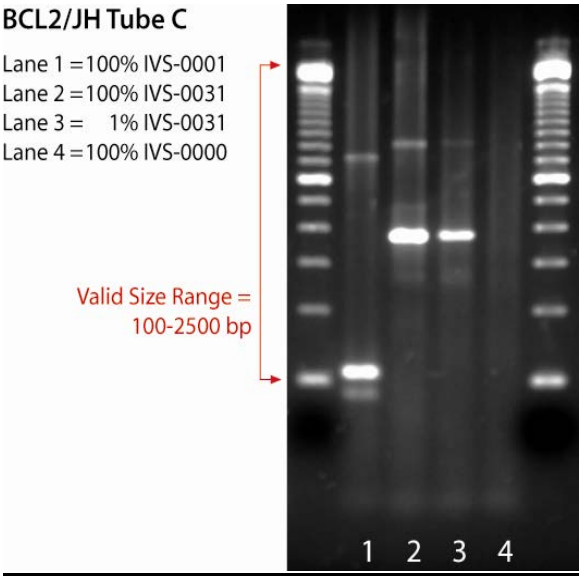

Figure 3. *BCL2*/JH Tube C master mix run on a 2% agarose gel.

20. *BCL2*/JH Translocation 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 of the master mixes). Aliquot volumes should be 45 µL for each sample + 135 µL for the positive, negative and no template controls. We recommend adding an additional 20 µL to correct for pipetting errors.
3. Add the appropriate amount of either AmpliTaq Gold, EagleTaq or AmpliTaq DNA polymerase (0.25 µL of either AmpliTaq Gold, EagleTaq or AmpliTaq @5 U/µL per 50 µL total reaction volume) to each of the master mixes and gently mix by inverting several times or gently vortexing.
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. Amplify target DNA using the universal thermal cycler program.

Gel Detection – Agarose TBE Gels

1. A 2% MetaPhor or NuSieve 3:1 agarose/TBE gel is prepared.
2. 20 µL from each of the amplification reactions are individually mixed with 4 µL of 6X gel loading buffer. 20 µL of this mixture is loaded into separate wells of the gel, flanked by DNA size standards. Products are detected using ethidium bromide or an equivalent dye.
3. Gel is photographed and data are interpreted.