A Sysmex Group Company

CytoCell

Instructions For Use

REF: LPH 108-S / LPH 108

IGH/MAF *Plus* v2 Translocation, Dual Fusion Probe

CE IVD

PROFESSIONAL USE ONLY

www.cytocell.com

Further information and other languages available at www.ogt.com

Limitations

This device is designed to detect rearrangements with breakpoints in the region bound by the red and green clones in this probe set, which includes the *IGH* and *MAF* regions. Breakpoints outside of this region, or variant rearrangements wholly contained within this region, may not be detected with this product.
The test is not intended for: use as a stand-alone diagnostic, prenatal testing, population-based screening, near-patient testing or self-testing. This product is intended for laboratory professional use only; all results should be interpreted by suitably-qualified staff, taking into account other relevant test results.
This product has not been validated for use on sample types or disease types other than those specified in the intended use.
Reporting and interpretation of FISH results should be consistent with professional standards of practice and should take into consideration other clinical and diagnostic information. This kit is intended as an adjunct to other diagnostic laboratory tests and therapeutic action should not be initiated on the basis of the FISH result alone.
Failure to adhere to the protocol may affect the performance and lead to false positive/negative results.
This kit has not been validated for purposes outside of the intended use stated.

Intended Use

The CytoCell IGH/MAF *Plus* v2 Translocation, Dual Fusion Probe is a qualitative, non-automated, fluorescence *in situ* hybridization (FISH) test used to detect chromosomal rearrangements between the 14q32.3 region on chromosome 14 and the 16q23 region on chromosome 16 in Carnoy's solution (3:1 methanol/acetic acid) fixed haematologically-derived cell suspensions from patients with confirmed or suspected multiple myeloma (MM).

Indications

This product is designed as an adjunct to other clinical and histopathological tests in recognised diagnostic and clinical care pathways, where knowledge of *IGH-MAF* translocation status would be important for clinical management.

Principles of the Test

Fluorescence *in situ* hybridisation (FISH) is a technique that allows DNA sequences to be detected on metaphase chromosomes or in interphase nuclei from fixed cytogenetic samples. The technique uses DNA probes that hybridise to entire chromosomes or single unique sequences, and serves as a powerful adjunct to G-banded cytogenetic analysis. This technique can now be applied as an essential investigative tool within prenatal, haematological and solid tumour chromosomal analysis. Target DNA, after fixation and denaturation, is available for annealing to a similarly denatured, fluorescently labelled DNA probe, which has a complementary sequence. Following hybridisation, unbound and non-specifically bound DNA probe is removed and the DNA is counterstained for visualisation. Fluorescence microscopy then allows the visualisation of the hybridised probe on the target material.

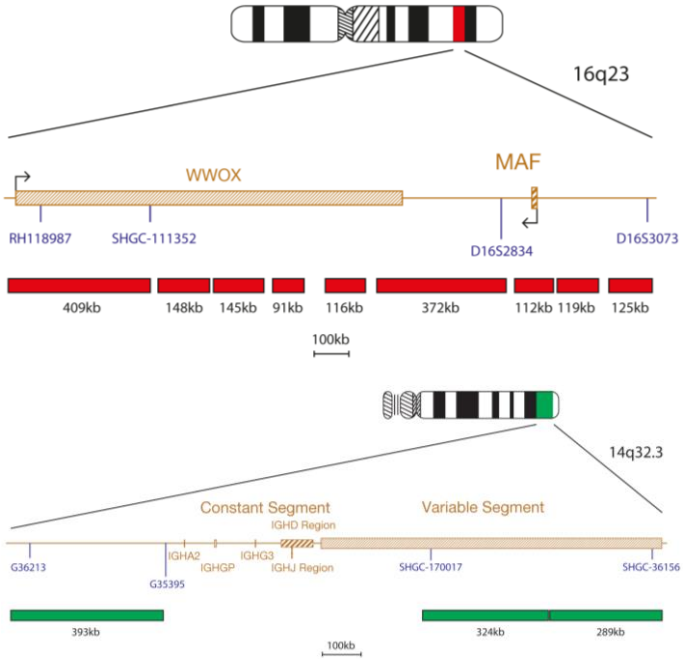
Probe Information

MAF (*MAF bZIP transcription factor*) gene is located at 16q23 and IGH (*immunoglobulin heavy locus*) at 14q32.3. Approximately 50-60% of multiple myeloma (MM) cases are associated with translocations involving IGH and one of several partners including *CCND1, NSD2 (WHSC1)* and *FGFR3, CCND3, MAF* or *MAFB*[1]. The t(14;16)(q32.3;q23) translocation is a recurrent translocation seen in 2-10% of MMs[1].

The majority of the breakpoints occur within the last intron of *WWOX* (*WW domain containing oxidoreductase*), centromeric to *MAF*. These breakpoints have a dual impact of positioning the IGH enhancer near *MAF* and disrupting the *WWOX* gene[2]. Gene expression profiling of myeloma cell lines revealed that *MAF* caused transactivation of cyclin D2 (a promoter of cell cycle progression), thus enhancing proliferation of myeloma cells[3].

According to the literature, MM patients harbouring the t(14;16) appear to have a more aggressive clinical outcome[4,5].

Probe Specification
MAF, 16q23, Red
IGH, 14q32.3, Green



The IGH/MAF *Plus* v2 Translocation, Dual Fusion Probe consists of the IGH probe mix, labelled in green, proximal to the Constant segment and within the Variable segment of the IGH region and the MAF probe mix, labelled in red, that encompasses the MAF gene and flanking regions as well as the WWOX gene.

Materials Provided

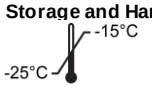
Probe: 50µl per vial (5 tests) or 100µl per vial (10 tests)
The probes are provided premixed in hybridisation solution (formamide; dextran sulphate; saline-sodium citrate (SSC)) and are ready to use.

Counterstain: 150µl per vial (15 tests)
The counterstain is DAPI antifade (ES: 0.125µg/ml DAPI (4,6-diamidino-2-phenylindole)).

Warnings and Precautions

1. For *in vitro* diagnostic use. For professional use only.
2. Wear gloves when handling DNA probes and DAPI counterstain.
3. Probe mixtures contain formamide, which is a teratogen; do not breathe fumes or allow skin contact. Handle with care; wear gloves and a lab coat.
4. DAPI is a potential carcinogen. Handle with care; wear gloves and a lab coat.
5. Dispose of all hazardous materials according to your institution's guidelines for hazardous waste disposal.
6. Operators must be capable of distinguishing the colours red, blue and green.
7. Failure to adhere to the outlined protocol and reagents may affect the performance and lead to false positive/negative results.
8. The probe should not be diluted or mixed with other probes.
9. Failure to use 10µl of probe during the pre-denaturation stage of the protocol may affect the performance and lead to false positive/negative results.

Storage and Handling



The kit should be stored between -25ºC to -15ºC in a freezer until the expiry date indicated on the kit label. The probe and counterstain vials must be stored in the dark.

The probe remains stable throughout the freeze-thaw cycles experienced during normal use (where one cycle constitutes the probe's removal from and replacement into the freezer) and is photostable for up to 48 hours after being exposed to continuous lighting conditions. All efforts must be made to limit exposure to light and temperature changes.

Equipment and Materials Necessary but not Supplied

Calibrated equipment must be used:

1. Hotplate (with a solid plate and accurate temperature control up to 80ºC)
2. Calibrated variable volume micropipettes and tips range 1µl - 200µl
3. Water bath with accurate temperature control at 37ºC and 72ºC
4. Microcentrifuge tubes (0.5ml)
5. Fluorescence microscope (Please see Fluorescence Microscope Recommendation section)
6. Phase contrast microscope
7. Clean plastic, ceramic or heat-resistant glass Coplin jars
8. Forceps
9. Calibrated pH meter (or pH indicator strips capable of measuring pH 6.5 – 8.0)
10. Humidified container
11. Fluorescence grade microscope lens immersion oil
12. Bench top centrifuge
13. Microscope slides
14. 24x24mm coverslips
15. Timer
16. 37ºC incubator
17. Rubber solution glue
18. Vortex mixer
19. Graduated cylinders
20. Magnetic stirrer
21. Calibrated thermometer

Optional Equipment not Supplied

1. Cytogenetic drying chamber

Reagents Needed but not Supplied

1. 20x saline-sodium citrate (SSC) Solution
2. 100% Ethanol
3. Tween-20
4. 1M Sodium hydroxide (NaOH)
5. 1M Hydrochloric acid (HCl)
6. Purified water

Fluorescence Microscope Recommendation

Use a 100-watt mercury lamp or equivalent and oil immersion plan apochromat objectives 60/63x or 100x for optimal visualisation. The fluorophores used in this probe set will excite and emit at the following wavelengths:

Fluorophore	$Excitation_{max}$ [nm]	$Emission_{max}$ [nm]
Green	495	521
Red	596	615

Ensure appropriate excitation and emission filters that cover the wavelengths listed above are fitted to the microscope. Use a triple bandpass DAPI/green spectrum/red spectrum filter or a dual bandpass green spectrum/red spectrum filter for optimal simultaneous visualisation of the green and red fluorophores.

Check the fluorescence microscope before use to ensure it is operating correctly. Use immersion oil that is suitable for fluorescence microscopy and formulated for low auto fluorescence. Avoid mixing DAPI antifade with microscope immersion oil as this will obscure signals. Follow manufacturers' recommendations in regards to the life of the lamp and the age of the filters.

Sample Preparation

The kit is designed for use on haematologically-derived cell suspensions fixed in Carnoy's solution (3:1 methanol/acetic acid) fixative, that are prepared according to the laboratory or institution guidelines. Prepare air dried samples on microscope slides according to standard cytogenetic procedures. The AGT *Cytogenetics Laboratory Manual* contains recommendations for specimen collection, culturing, harvesting and for slide making[7].

Solution Preparation

Ethanol Solutions

Dilute 100% ethanol with purified water using the following ratios and mix thoroughly:

- 70% Ethanol - 7 parts 100% ethanol to 3 parts purified water
- 85% Ethanol - 8.5 parts 100% ethanol to 1.5 parts purified water

Store the solutions for up to 6 months at room temperature in an airtight container.

2xSSC Solution

Dilute 1 part 20xSSC Solution with 9 parts purified water and mix thoroughly. Check pH and adjust to pH 7.0 using NaOH or HCl as required. Store the solution for up to 4 weeks at room temperature in an airtight container.

0.4xSSC Solution

Dilute 1 part 20xSSC Solution with 49 parts purified water and mix thoroughly. Check pH and adjust to pH 7.0 using NaOH or HCl as required. Store the solution for up to 4 weeks at room temperature in an airtight container.

2xSSC, 0.05% Tween-20 Solution

Dilute 1 part 20xSSC Solution with 9 parts purified water. Add 5µl of Tween-20 per 10ml and mix thoroughly. Check pH and adjust to pH 7.0 using NaOH or HCl as required. Store the solution for up to 4 weeks at room temperature in an airtight container.

FISH Protocol

(Note: Ensure that exposure of the probe and counterstain to laboratory lights is limited at all times).

Slide Preparation

1. Spot the cell sample onto a glass microscope slide. Allow to dry. (**Optional, if using a cytogenetic drying chamber**: slides should be spotted using a cytogenetic drying chamber. The chamber should be operated at approximately 25°C and 50% humidity for optimal cell sample spotting. If a cytogenetic drying chamber is not available, use a fume hood as an alternative).
2. Immerse the slide in 2xSSC for 2 minutes at room temperature (RT) without agitation.
3. Dehydrate in an ethanol series (70%, 85% and 100%), each for 2 minutes at RT.
4. Allow to dry.

Pre-Denaturation

5. Remove the probe from the freezer and allow it to warm to RT. Briefly centrifuge tubes before use.
6. Ensure that the probe solution is uniformly mixed with a pipette.
7. Remove 10µl of probe per test, and transfer it to a microcentrifuge tube. Quickly return the remaining probe to the freezer.
8. Place the probe and the sample slide to prewarm on a 37ºC (+/- 1ºC) hotplate for 5 minutes.
9. Spot 10µl of probe mixture onto the cell sample and carefully apply a coverslip. Seal with rubber solution glue and allow the glue to dry completely.

Denaturation

10. Denature the sample and probe simultaneously by heating the slide on a hotplate at 75ºC (+/- 1ºC) for 2 minutes.

Hybridisation

11. Place the slide in a humid, lightproof container at 37ºC (+/- 1ºC) overnight.

Post-Hybridisation Washes

12. Remove the DAPI from the freezer and allow it to warm to RT.
13. Remove the coverslip and all traces of glue carefully.
14. Immerse the slide in 0.4xSSC (pH 7.0) at 72ºC (+/- 1ºC) for 2 minutes without agitation.
15. Drain the slide and immerse it in 2xSSC, 0.05% Tween-20 at RT (pH 7.0) for 30 seconds without agitation.
16. Drain the slide and apply 10µl of DAPI antifade onto each sample.
17. Cover with a coverslip, remove any bubbles and allow the colour to develop in the dark for 10 minutes.
18. View with a fluorescence microscope (see **Fluorescence Microscope Recommendation**).

Stability of Finished Slides

Finished slides remain analysable for up to 1 month if stored in the dark at/or below RT.

Procedural Recommendations

1. Baking or ageing of slides may reduce signal fluorescence
2. Hybridisation conditions may be adversely affected by the use of reagents other than those provided or recommended by Cytocell Ltd.
3. Use a calibrated thermometer for measuring temperatures of solutions, waterbaths and incubators as these temperatures are critical for optimum product performance.
4. The wash concentrations, pH and temperatures are important as low stringency can result in non-specific binding of the probe and too high stringency can result in a lack of signal.
5. Incomplete denaturation can result in lack of signal and over denaturation can also result in non-specific binding.
6. Over hybridisation can result in additional or unexpected signals.
7. Users should optimise the protocol for their own samples prior to using the test for diagnostic purposes.
8. Suboptimal conditions may result in non-specific binding that may be misinterpreted as a probe signal.

Interpretation of Results

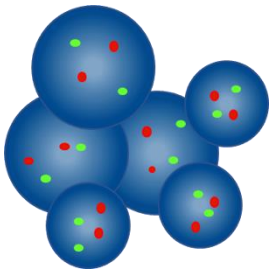
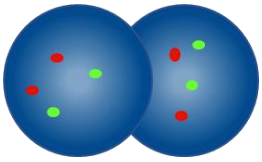
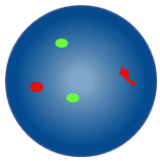
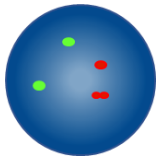
Assessing Slide Quality

The slide should not be analysed if:

- Signals are too weak to analyse in single filters - in order to proceed with analysis, signals should appear bright, distinct and easily evaluable
- There are high numbers of clumped/overlapping cells obstructing the analysis
- >50% of the cells are not hybridised
- There is excess of fluorescent particles between cells and/or a fluorescent haze that interferes with the signals - in optimal slides the background should appear dark or black and clean
- Cell nucleus borders cannot be distinguished and are not intact

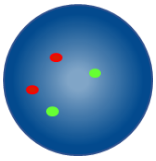
Analysis Guidelines

- Two analysts should analyse and interpret each sample. Any discrepancies should be resolved by assessment by a third analyst
- Each analyst should be suitably qualified according to recognised national standards
- Each analyst should score independently 100 nuclei for each sample. The first analyst should start the analysis from the left side of the slide and the second analyst from the right one
- Each analyst should document their results in separate sheets
- Analyse only intact nuclei, not overlapped or crowded nuclei or nuclei covered by cytoplasmic debris or high degree of autofluorescence
- Avoid areas where there is excess of cytoplasmic debris or non-specific hybridisation
- Signal intensity may vary, even with a single nucleus. In such cases, use single filters and/or adjust the focal plane
- In suboptimal conditions signals may appear diffuse. If two signals of the same colour touch each other, or the distance between them is no greater than two signal widths, or when there is a faint strand connecting the two signals, count as one signal
- If in doubt about whether a cell is analysable or not, then do not analyse it

Analysis Guidelines	
	Do not count – nuclei are too close together to determine boundaries
	Do not count overlapping nuclei – all areas of both nuclei are not visible
	Count as two red signals and two green signals – one of the two red signals is diffuse
	Count as two red signals and two green signals – the gap in one red signal is less than two probe widths

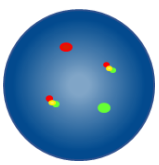
Expected Results

Expected Normal Signal Pattern



In a normal cell, two red and two green signals (2R, 2G) are expected.

Expected Abnormal Signal Pattern



In a cell with a t(14;16)(q32.3;q23) translocation, the expected signal pattern will be one red, one green and two fusion signals (1R, 1G, 2F).

Other signal patterns are possible in aneuploid/unbalanced specimens. Please note that in the presence of other IGH rearrangements apart from the IGH/MAF translocation the green IGH signal may appear split.

Known Cross-Reactivity

The green IGH probe may show cross-hybridisation to 15q11.2 and 16p11.2.

Adverse Event Reporting

If you believe this device has malfunctioned or suffered a deterioration in its performance characteristics which may have contributed to an adverse event (e.g. delayed or misdiagnosis, delayed or inappropriate treatment), this must be reported immediately to the manufacturer (**email:** vigilance@ogt.com).

If applicable, the event should also be reported to your national competent authority. A list of vigilance contact points can be found at: http://ec.europa.eu/growth/sectors/medical-devices/contacts/.

Specific Performance Characteristics

Analytical Specificity

Analytical specificity is defined as the percentage of signals that hybridise to the correct locus and no other location. Four chromosomal loci in each of twenty metaphase cells from five samples were analysed, giving 400 data points. The location of each hybridised probe was mapped and the number of metaphase chromosome FISH signals that hybridised to the correct locus was recorded.

The analytical specificity of each probe in the kit was calculated as the number of metaphase chromosome FISH signals hybridised to the correct locus divided by the total number of metaphase chromosome FISH signals hybridised, this result was multiplied by 100, expressed as a percentage and given with a 95% confidence interval.

Table 1. Analytical Specificity for the IGH/MAF v2 Translocation, Dual Fusion Probe

Target	Number of metaphase chromosomes hybridised	Number of correctly hybridised loci	Analytical Specificity	95% Confidence Interval
14q32.3	200	200	100%	98.12% - 100%
16q23	200	200	100%	98.12% - 100%

Analytical Sensitivity

Analytical sensitivity is the percentage of scoreable interphase cells with the expected normal signal pattern. A minimum of 200 interphase cells were analysed for each of 25 karyotypically normal fixed bone marrow samples or bone marrow samples negative for an IGH rearrangement and 25 IGH negative CD138+ cell samples, resulting in a minimum of 5000 nuclei scored for each sample type. The sensitivity data was analysed based on the percentage of cells showing a normal expected signal pattern and expressed as a percentage with a 95% confidence interval.

Table 2. Analytical Sensitivity for the IGH/MAF v2 Translocation, Dual Fusion Probe

Sample Type	Sensitivity Criteria	Sensitivity Result
Bone Marrow	>95%	98.76% ± 0.55%
CD138+	>95%	96.64% ± 1.17%

Characterisation of Normal Cut-off Values

The normal cut-off is defined as the percentage of cells exhibiting a false positive signal pattern at which an individual would be considered normal and not consistent with a clinical diagnosis. A minimum of 200 interphase cells were analysed for each of 25 karyotypically normal fixed bone marrow samples or bone marrow samples negative for an IGH rearrangement and 25 IGH negative CD138+ cell samples, resulting in a minimum of 5000 nuclei scored for each sample type.

The cut-off value was determined using the β-inverse (BETAINV) function in MS Excel. It was calculated as the percentage of interphase cells showing a false positive signal pattern using the upper bound of a one-sided 95% confidence interval of the binomial distribution in a normal patient sample.

Table 3. Characterisation of Normal Cut-off Values for the IGH/MAF v2 Translocation, Dual Fusion Probe

Sample Type	Cut-off Result
Bone Marrow	1.5%
CD138+	2.5%

Laboratories must verify cut-off values using their own data[7,8].

Precision

The precision of this product has been measured in terms of intra-day precision (sample-to-sample), inter-day precision (day-to-day) and single-site inter-lot precision (lot-to-lot).

Three samples were used to assess the precision of this product: one contrived normal bone marrow sample (pooled from 25 individual samples), one contrived normal CD138+ sample (pooled from 28 individual samples) and one low positive CD138+ sample (2-4x the product's cut-off, created by spiking the normal CD138+ sample with a known positive), which was used to challenge the product around the established cut-off.

To establish the inter-day and intra-day precision, the samples were evaluated over five non-consecutive dates and to establish the lot-to-lot precision, three lots of the

product were evaluated on four replicates of the same samples. The results were presented as the overall agreement with the predicted negative class (for the negative samples).

Table 4. Reproducibility and Precision for the IGH/MAF v2 Translocation, Dual Fusion Probe

Variable	Sample type	Agreement
Intra-day and inter-day precision	Normal bone marrow (negative)	100%
	Normal CD138+ (negative)	100%
	Low positive CD138+	100%
Lot-to-lot precision	Normal bone marrow (negative)	100%
	Normal CD138+ (negative)	100%
	Low positive CD138+	100%

Clinical Performance

To ensure that the product detects intended rearrangements, clinical performance was established over two studies on representative samples of the intended population for the product: one utilising CD138+ specimens and one utilising bone marrow specimens. The sample size for each study was twenty specimens, with the target population of five IGH-MAF fusion positive specimens and fifteen IGH-MAF fusion negative specimens. All samples were de-identified and randomised to prevent analysis bias. The results were compared to the known status of the sample. The probe correctly identified the status of the samples in all instances.

The results of these tests were analysed in order to provide clinical sensitivity, clinical specificity and false positive rate (FPR) values for positive signals, using a one-dimensional approach.

Table 5. Clinical Performance for the IGH/MAF v2 Translocation, Dual Fusion Probe

Variable	Result
Clinical Sensitivity (true positive rate, TPR)	98.1%
Clinical Specificity (true negative rate, TNR)	100%
False Positive rate (FPR) = 1 – Specificity	0%

Additional Information

For additional product information please contact the CytoCell Technical Support Department.
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References

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2. Walker *et al.*, Blood 2013;121(17);3413-3419
3. Chang H *et al.*, Leukemia 2007;21:1572-1574
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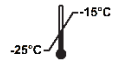
7. Mascarello JT, Hirsch B, Kearney HM, et al. Section E9 of the American College of Medical Genetics technical standards and guidelines: fluorescence in situ hybridization. Genet Med. 2011;13(7):667-675.
8. Wiktor AE, Dyke DLV, Stupca PJ, Ketterling RP, Thorland EC, Shearer BM, Fink SR, Stockero KJ, Majorowicz JR, Dewald GW. *Preclinical validation of fluorescence in situ hybridization assays for clinical practice*. Genetics in Medicine. 2006;8(1):16–23.

Guide to Symbols

REF	**en:** Catalogue number
IVD	**en:** *In vitro* diagnostic medical device
LOT	**en**: Batch code
	**en:** Consult instructions for use
	**en:** Manufacturer
	**en:** Use-by date
	**en:** Temperature limit
	**en:** Keep away from sunlight
	**en:** Contains sufficient for <n> tests
CONT	**en:** Contents

Patents and Trademarks

CytoCell is a registered trademark of Cytocell Ltd.

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