FAST PML/RARα (RARA) Translocation, Dual Fusion Probe

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Probes Information

The PML (promyelocytic leukemia) gene is located at 15q24.1 and the RARA (retinoic acid receptor alpha) gene is located at 17q21.2. The translocation t(15;17)(q24;q21) gives rise to the PML-RARA fusion gene and is the diagnostic hallmark of acute promyelocytic leukaemia (APL).

This FAST PML/RARA FISH probe allows rapid detection of the rearrangement, with only one hour of hybridisation required.

The PML-RARA fusion gene is created by the t(15;17)(q24;q21) translocation, found in more than 90% of cases of APL, a leukaemia that comprises 5-8% of cases of acute myeloid leukaemia (AML). In a subset of cases, variant RARA translocations can be observed. Known fusion partners include NPM1 at 5q35, NUMA1 at 11q13, ZBTB16 (PLZF) at 11q23, STAT5B at 17q21, PRKAR1A at 17q24, FIP1L1 at 4q12 and BCCOR at Xp11.22-24.

PML and RARA have both been implicated in normal haematopoiesis. PML possesses growth suppressor and proapoptotic activity whereas RARA is a transcription factor that mediates the effect of retinoic acid at specific response elements. PML-RARA fusion protein behaves as an altered retinoic acid receptor with an ability of transmitting oncogenic signalling.

Immediate treatment of APL patients is critical, due to fatality coagulation disorders and life-threatening haemorrhage in diagnosis. Prior to the introduction of all-trans-retinoic-acid (ATRA) and arsenic trioxide (ATO) in APL treatment protocols, the disease had a poor prognosis; however, since the introduction of these therapies, the overall survival rate has improved dramatically, with nearly 90% of patients cured. Patients with variant RARA translocations show variable sensitivity to treatment, with some patients showing resistance to treatment protocols. It is therefore important to differentiate between APL patients with PML-RARA fusion and those patients with variant RARA translocations.

Probe Specification

PML, 15q24, Red
RARα, 17q21.1-1.2, Green

The PML-RARA fusion gene is created by the t(15;17)(q24;q21) translocation, found in more than 90% of cases of APL, a leukaemia that comprises 5-8% of cases of acute myeloid leukaemia (AML).

The PML probe mix, labelled in red, consists of a 153kb probe centromeric to the PML gene that covers the marker D15S169 and a 176kb probe telomeric to the PML gene that covers the marker D15S965. The PML - RARA fusion gene is created by the t(15;17)(q24;q21) translocation, found in more than 90% of cases of APL, a leukaemia that comprises 5-8% of cases of acute myeloid leukaemia (AML). The probes are provided premixed in hybridisation solution (formamide; dextran sulphate; saline-sodium citrate (SSC)) and are ready to use.

Indications

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

Materials Provided

Probe: 50µl per vial (5 tests) or 100µl per vial (10 tests)
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
-15°C

The kit should be stored between -25°C to -15°C 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 photosstable 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 37°C and 72°C
4. Microcentrifuge tubes (0.5ml)
5. 1M Hydrochloric acid (HCl)
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. 24x24mm coverslips
14. 0.4xSSC Solution
15. Microscope slides
16. 0.1µl bruising pipette
17. Vortex mixer
18. 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:

<table>
<thead>
<tr>
<th>Fluorophore</th>
<th>Excitation max [nm]</th>
<th>Emission max [nm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Green</td>
<td>495</td>
<td>521</td>
</tr>
<tr>
<td>Red</td>
<td>595</td>
<td>615</td>
</tr>
</tbody>
</table>

Ensure appropriate excitation and emission filters that cover the wavelengths listed above are fitted to the microscope. Use a triple bandpass DAPI/green 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 autofluorescence. 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 cytogenetics procedures. The AGT Cytogenetics Laboratory Manual contains recommendations for specimen collection, culturing, harvesting and for slide making.

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.

FAST FISH Protocol – One (1) hour hybridisation
(Not: 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: 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.
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 37°C (+/- 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 75°C (+/- 1°C) for 2 minutes.

Hybridisation
11. Place the slide in a humid, lightproof container at 37°C (+/- 1°C) for one (1) hour.

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. Immers the slide in 0.4xSSC (pH 7.0) at 72°C (+/- 1°C) 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).

Standard FISH Protocol – Overnight hybridisation
(Not: 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: 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.
8. Place the probe and the sample slide to prewarm on a 37°C (+/- 1°C) hotplate for 5 minutes.
9. Spot 10µl of probe mixture onto the cell sample and carefully apply a coverslip.

Denaturation
10. Denature the sample and probe simultaneously by heating the slide on a hotplate at 75°C (+/- 1°C) 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 (+/- 1°C) 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 signal widths

Expected Results

Expected Normal Signal Pattern

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

Expected Abnormal Signal Patterns

In a cell with a t(15;17)(q24.1;q21) translocation, the expected signal pattern will be one red, one green and two fusions (1R, 1G, 2F).

Other signal patterns are possible in aneuploid/unbalanced specimens.

Known Cross-Reactivity
No known cross-reactivity.

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 the percentage of signals that hybridise to the correct locus and no other location. The analytical specificity was established by analysing a total of 200 target loci. The analytical specificity was calculated as the number of FISH signals that hybridised to the correct locus divided by the total number of FISH signals hybridised.
The normal cut-off value. The results were then compared to the known status of the interphase cells were recorded. A normal/abnormal determination was made by intended population for the product. For each sample, the signal patterns of the clinical performance was established on a representative sample of the product.

**Analytical Sensitivity**

Analytical sensitivity is the percentage of scoreable interphase cells with the expected normal signal pattern. The analytical sensitivity was established by analysing interphase cells across different normal samples. The sensitivity was calculated as the percentage of scoreable cells with the expected signal pattern (with a 95% confidence interval).

The normal cut-off value was established using samples from normal and positive samples. The sensitivity was calculated as the percentage of scoreable interphase cells with the expected abnormal signal pattern at which a sample is considered normal for that signal pattern.

The Youden index was calculated to find the threshold value for which Sensitivity + Specificity-1 is maximised.

Laboratories must verify cut-off values using their own data9,10.

**Precision and Reproducibility**

Precision is a measure of the natural variation of a test when repeated several times under the same conditions. This was assessed by analysing repeats of the sample. The reproducibility is a measure of variability of a test and has been established in terms of sample-to-sample, day-to-day and batch-to-batch variability. Day-to-day reproducibility was assessed by analysing the same samples on three different days. Batch-to-batch reproducibility was assessed by analysing the same samples using three different lot numbers of probe on one day. Sample-to-sample reproducibility was assessed by analysing three replicates of a sample on one day. For each sample, signal patterns of 100 interphase cells were recorded and the percentage of cells with the expected signal pattern was calculated.

The reproducibility and precision were calculated as the Standard Deviation (STDEV) between replicates for each variable and overall mean STDEV.

The normal cut-off value was established using samples from normal and positive samples. For each sample, the signal patterns of 100 cells were recorded. The Youden index was calculated to find the threshold value for which Sensitivity + Specificity-1 is maximised.

Laboratories must verify cut-off values using their own data9,10.

**Precision and Reproducibility**

Precision is a measure of the natural variation of a test when repeated several times under the same conditions. This was assessed by analysing repeats of the sample. The reproducibility is a measure of variability of a test and has been established in terms of sample-to-sample, day-to-day and batch-to-batch variability. Day-to-day reproducibility was assessed by analysing the same samples on three different days. Batch-to-batch reproducibility was assessed by analysing the same samples using three different lot numbers of probe on one day. Sample-to-sample reproducibility was assessed by analysing three replicates of a sample on one day. For each sample, signal patterns of 100 interphase cells were recorded and the percentage of cells with the expected signal pattern was calculated.

The reproducibility and precision were calculated as the Standard Deviation (STDEV) between replicates for each variable and overall mean STDEV.

**Clinical Performance**

The clinical performance was established on a representative sample of the intended population for the product. For each sample, the signal patterns of 100 interphase cells were recorded. A normal/abnormal determination was made by comparing the percentage of cells with the specific abnormal signal pattern to the normal cut-off value. The results were then compared to the known status of the sample.

The results of the clinical data were analysed in order to produce sensitivity, specificity and cut off values using a one-dimensional approach.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Target Locus</th>
<th>No. of Signals Hybridised to the Correct Locus</th>
<th>Total No. of Signals Hybridised</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red PML</td>
<td>15q24.1</td>
<td>200</td>
<td>200</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Green RARα</td>
<td>17q21</td>
<td>200</td>
<td>200</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

**Analytical Sensitivity**

The abnormal signal pattern, in association with FISH probes, is the maximum percentage of scoreable interphase cells with a specific abnormal signal pattern at which a sample is considered normal for that signal pattern.

<table>
<thead>
<tr>
<th>Abnormal signal pattern</th>
<th>Youden Index</th>
<th>Normal Cut-off (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1R, 1G, 2F</td>
<td>1.0</td>
<td>4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Variable</th>
<th>Standard Deviation (STDEV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precision</td>
<td>0.00</td>
</tr>
<tr>
<td>Sample-to-sample</td>
<td>0.00</td>
</tr>
<tr>
<td>Day-to-day</td>
<td>0.00</td>
</tr>
<tr>
<td>Batch-to-batch</td>
<td>0.00</td>
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<tr>
<td>Overall deviation</td>
<td>0.00</td>
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**References**


**Guide to Symbols**

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<thead>
<tr>
<th>REF</th>
<th>en: Catalogue number</th>
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<tbody>
<tr>
<td>IVD</td>
<td>en: In vitro diagnostic medical device</td>
</tr>
<tr>
<td>LOT</td>
<td>en: Batch code</td>
</tr>
<tr>
<td>CONSULT</td>
<td>en: Consult instructions for use</td>
</tr>
<tr>
<td>MANUFACTURER</td>
<td>en: Manufacturer</td>
</tr>
<tr>
<td>USE-BY-DATE</td>
<td>en: Use-by date</td>
</tr>
<tr>
<td>TEMPERATURE LIMIT</td>
<td>en: Temperature limit</td>
</tr>
<tr>
<td>KEEP AWAY FROM SUNLIGHT</td>
<td>en: Keep away from sunlight</td>
</tr>
<tr>
<td>CONTAINS SUFFICIENT FOR (n) TESTS</td>
<td>en: Contains sufficient for (n) tests</td>
</tr>
</tbody>
</table>

**Table 1. Analytical Specificity for the FAST PML/RARα Translocation, Dual Fusion Probe**

<table>
<thead>
<tr>
<th>Catalogue number</th>
<th>Manufacturer</th>
<th>Contents</th>
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<tbody>
<tr>
<td>DS211/CE-en v006.00/2021-06-11 (H043 v3 / H044 v3)</td>
<td>Cytocell Ltd.</td>
<td>Cytocell Ltd. Oxford Gene Technology, 418 Cambridge Science Park, Milton Road, Cambridge, CB4 0PZ, UK T: +44(0)1223 294986 F: +44(0)1223 294986 E: <a href="mailto:probes@cytocell.com">probes@cytocell.com</a> W: <a href="http://www.ogt.com">www.ogt.com</a></td>
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