A Sysmex Group Company

CytoCell

Instructions For Use

REF: USA-LPH026

AML1/ETO (RUNX1/RUNX1T1) Translocation, Dual Fusion FISH Probe Kit

IVD

PROFESSIONAL USE ONLY

Further information available at www.ogt.com

Intended Use

The AML1/ETO (RUNX1/RUNX1T1) Translocation, Dual Fusion FISH Probe Kit is a fluorescence in situ hybridization (FISH) Test used to detect rearrangement involving the *AML1* (*RUNX1*) region on chromosome 21 at location 21q22.1 and the *ETO* (*RUNX1T1*) region on chromosome 8 at location 8q21.3 in fixed bone marrow specimens from patients with acute myeloid leukemia (AML). The test is indicated for characterization of patient specimens consistent with World Health Organization (WHO) guidelines for Classification of Tumours of Haematopoietic and Lymphoid Tissues (Revised 4th Edition) and in conjunction with other clinicopathological criteria. The assay results are intended to be interpreted by a qualified pathologist or cytogeneticist. The test is not intended for use as a stand-alone diagnostic, disease screening, or as a companion diagnostic.

Principles of the test

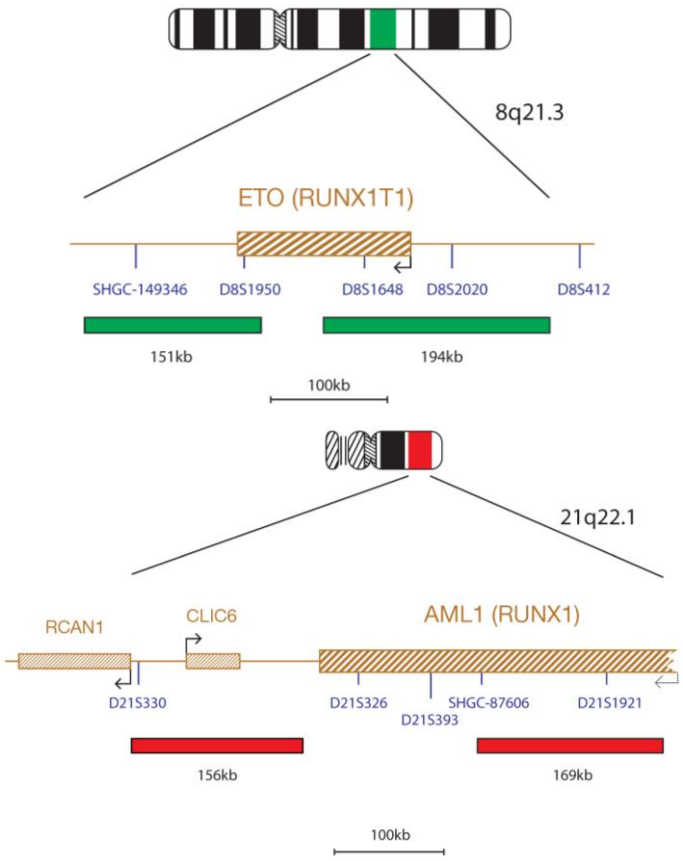
Fluorescence *in situ* hybridization (FISH) is a technique that allows DNA sequences to be detected on metaphase chromosomes or in interphase nuclei from fixed cytogenetic samples. The technique uses DNA probes that hybridize to entire chromosomes or single unique sequences and serves as a powerful adjunct to G-banded cytogenetic analysis. This technique can now be applied as an essential investigative tool within prenatal, hematological and solid tumor 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 hybridization, unbound and non-specifically bound DNA probe is removed and the DNA is counterstained for visualization. Fluorescence microscopy then allows the visualization of the hybridized probe on the target material.

Probe Information

AML with a *RUNX1-RUNX1T1* fusion resulting from a t(8;21)(q22;q22) translocation is a recognized disease entity according to the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia. The translocation is commonly observed in patients with AML FAB type M2, most commonly in children and young adults and is a good prognostic indicator. The t(8;21) breakpoint mainly occurs in the intron between exons 5 and 6, just before the transactivation domain. The fusion protein created contains the DNA-binding domain of *RUNX1* fused to the transcription factor *RUNX1T1*. In addition to the reciprocal t(8;21) translocation creating the *RUNX1-RUNX1T1* fusion, variant translocations have also been reported. These variant rearrangements may be cryptic and easily overlooked by G-banding; however, FISH can indicate the presence of such rearrangements.

Probe Specification

ETO, 8q21.3, FITC green.
AML1, 21q22.1, Texas Red.



The AML1/ETO (RUNX1/RUNX1T1) Translocation, Dual Fusion FISH Probe Kit consists of a 156kb probe labeled in Texas Red, centromeric to the *AML1* (*RUNX1*) gene, including the *CLIC6* gene; a 169kb probe labelled in Texas red, telomeric to *AML1* (*RUNX1*) gene, extending beyond the marker D21S1921; and two (151kb and 194kb) probes, labeled inn FITC green, on either side of the *ETO* (*RUNX1T1*) gene.

Materials Provided

Probe: 100µl per vial (LPH026: 10 tests)
Amount of Texas Red AML1 probe: 14.0-23.6 ng/test
Amount of FITC green ETO probe: 82.2-123.0 ng/test
The probes are provided premixed in hybridization solution (formamide; dextran sulfate; saline-sodium citrate (SSC)) and are ready to use.

Counterstain: 150µl per vial (DES150L**:** 15 tests)
The counterstain is DAPI antifade (0.125µg/ml DAPI (4,6-diamidino-2-phenylindole)).

Warnings and Precautions

1. For *in vitro* diagnostic use. For professional use only.
2. For prescription use only
3. Wear gloves when handling DNA probes and DAPI counterstain.
4. Probe mixtures contain formamide, which is a teratogen; do not breathe fumes or allow skin contact. Wear gloves, a lab coat, and handle in a fume hood. Upon disposal, flush with a large volume of water.
5. DAPI is a potential carcinogen. Handle with care; wear gloves and a lab coat. Upon disposal, flush with a large volume of water.
6. Dispose of all hazardous materials according to your institution's guidelines for hazardous waste disposal.
7. Operators must be capable of distinguishing the colors red, blue and green.
8. The probe is intended to be used only at the concentration provided and is not intended to be diluted
9. Failure to adhere to the protocol and the reagents may affect the performance and lead to false positive/negative results.

Labelling according to GHS-US hazard label requirements

Hazard pictograms (GHS-US):

GHS07 GHS08

Signal word (GHS-US): Danger

Hazardous ingredients: Formamide <100%

Hazard statements (GHS-US):

H315 - Causes skin irritation
H319 - Causes serious eye irritation
H360 - May damage fertility or the unborn child

Precautionary statements (GHS-US):
P202 - Do not handle until all safety precautions have been read and understood
P280 - Wear eye protection, protective clothing, protective gloves
P302+P352 - IF ON SKIN: Wash with plenty of soap and water
P305+P351+P338 - IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing
P308+P313 - IF exposed or concerned: Get medical advice/attention
P362+P364 - Take off contaminated clothing and wash it before reuse
P501 - Dispose of contents/container to hazardous or special waste collection point, in accordance with local, regional, national and/or international regulation

Refer to the Safety Data Sheet for more information.

Storage and Handling
Store the kit at -20ºC until the expiry date indicated on the kit label. Store the probe and counterstain vials in the dark. Ensure that exposure of the probe and counterstain to laboratory lights is limited at all times.

Equipment and Materials Necessary but not Supplied
1. Hotplate (with a solid plate and accurate temperature control up to 80ºC).
2. 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. Centrifuge
6. Fluorescence microscope (Please see Fluorescence Microscope Recommendation section).
7. Plastic or heat-resistant glass Coplin jars.
8. Forceps.
9. 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. Superfrost microscope slides.
14. 24x24mm coverslips or equivalent.
15. Timer.
16. 37ºC incubator.
17. Rubber solution glue.
18. Vortex mixer.
19. Graduated cylinders.
20. Magnetic stirrer.
21. Calibrated thermometer.
22. Clear nail varnish.

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.
7. Immersion oil.

Fluorescence Microscope Recommendation
Use a 100-watt mercury lamp, or equivalent, and oil immersion plan apochromat objectives x63 or x100 for optimal visualization. Use a triple bandpass filter DAPI/FITC/Texas Red or a dual bandpass filter FITC/Texas Red for optimal simultaneous visualization of the green and red fluorophores. The aqua fluorophore has specificity to the aqua and DEAC spectrum (single bandpass aqua or DEAC filter is required).
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 with regard to the life of the lamp and the age of the filters.

Sample Preparation
The kit is designed for use on bone marrow cells 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.

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.

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.

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.

2xSSC, 0.05%Tween-20
Dilute 1 part 20xSSC Solution with 9 parts purified water. Add 5µl of Tween-20 per 10ml of diluted SSC solution 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.

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.
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 room temperature (RT). Briefly centrifuge tubes before use.
6. Ensure that the probe solution is uniformly mixed with a pipette or a vortex mixer.
7. Remove 10µl of probe per test, and transfer it to a microcentrifuge tube. Quickly return the remaining probe to -20ºC.
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.

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

Post-Hybridization Washes
12. Remove the DAPI from the freezer and allow it to warm to room temperature (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 24x24mm coverslip, remove any bubbles.
18. Edge the slide with clear nail varnish to seal.
19. Allow the color to develop in the dark for 10 minutes.
20. View with a fluorescence microscope. (See **Fluorescence Microscope Recommendation**.)

Stability of Finished Slides
Hybridized slides remain analyzable for up to 1 month if stored in the dark at refrigerator temperature.

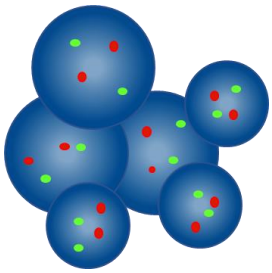
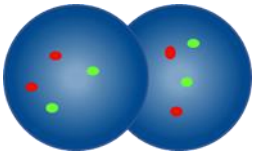
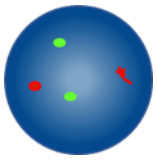
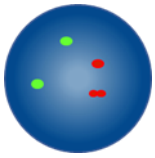
Procedural Recommendations
1. Baking or ageing of slides may reduce signal fluorescence
2. Hybridization conditions may be adversely affected by the use of reagents other than those provided or recommended by Cytocell Ltd.
3. Use a calibrated thermometer for measuring temperatures of solutions, waterbaths 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 hybridization can result in additional or unexpected signals.
7. Users should optimize 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 analyzed if:
- Signals are too weak to analyze 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 hybridized
- There is excess of fluorescent particles between cells and/or a fluorescent haze - 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 analyze each sample
- Each analyst should score independently 100 nuclei for each sample. The first analyst should start the analysis from the left side of the spot and the second analyst from the right one, so that the analysts are examining different areas of the hybridization and not scoring the same cells.
- Each analyst should document their results in separate sheets.
- Analyze 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 hybridization.

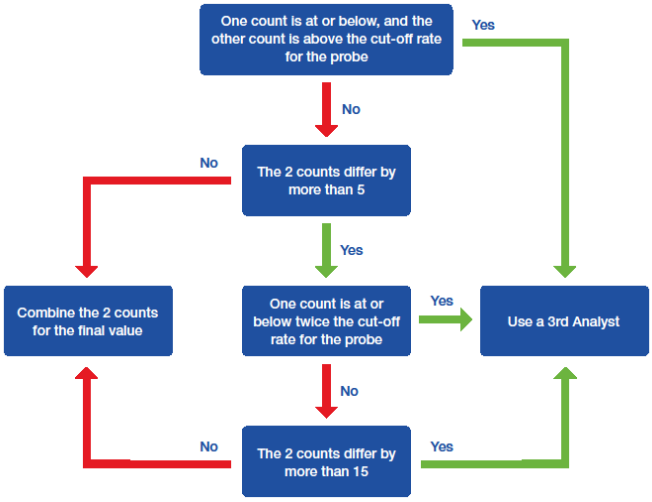
- Signal intensity may vary, even with a single nucleus. In such cases, use single filters and/or adjust the focal plane.
- In suboptimal conditions signals may appear diffuse. If two signals of the same color touch each other, or the distance between them is no greater than one signal width, or when there is a faint strand connecting the two signals, count as one signal
- If in doubt about whether a cell is analyzable or not, then do not analyze 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

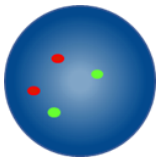
Third analyst requirements
Each of the two analysts will analyze 100 cells. In some cases, depending on the number of abnormal nuclei each analyst has seen, a third reader may be required. The rules for a third analyst are detailed in the text and the flowchart below.

A third analyst is required if:
- One of the analyst's abnormal signal count is above the cut-off and the other below or at the cut-off.
- The two-analysts' abnormal counts differ by more than 5 and either analyst's abnormal signal count is equal to or lower than twice the cut off.
- The two analysts' abnormal signal patterns differ by more than 15.

The third analyst will also analyze 100 nuclei. Of the three scores obtained, the two that are closer to each other should be used. If the 3 scores are equidistant, then the median value should be doubled and used.

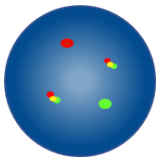


Expected Results
Expected normal signal pattern



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

Expected abnormal signal pattern



In a cell with a t(8;21)(q22;q22) 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.

Limitations of the procedure
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 results.

Each lab is responsible for establishing their own cut-off values. Each laboratory should test sufficiently large number of samples to establish normal population distribution of the signal levels and to assign a cut-off value. The product is for professional use only and is intended to be interpreted by a qualified Pathologist or Cytogeneticist.

The device has not been specifically validated in patients with <20% blast count.

Specific Performance Characteristics

Analytical Specificity
Analytical specificity is the percentage of signals that hybridize to the correct locus and no other location. The analytical specificity of the AML1/ETO (RUNX1/RUNX1T1) Translocation, Dual Fusion FISH Probe Kit was established by analyzing a total of 200 target loci from metaphase chromosomes prepared from five normal male peripheral blood samples. The analytical specificity was calculated as the number of FISH signals that hybridized to the correct locus divided by the total number of FISH signals hybridized. The result was then multiplied by 100, to give a percentage, and is given with a 95% confidence interval. The analytical specificity of the AML1/ETO (RUNX1/RUNX1T1) Translocation, Dual Fusion FISH Probe Kit probes was 100%, as shown in Table 1.

Table 1. Analytical Specificity for the AML1/ETO (RUNX1/RUNX1T1) Translocation, Dual Fusion FISH Probe Kit

Probe	Target	Number of metaphase chromosomes hybridized	Number of correct hybridized loci	Analytical Specificity (%)	95% Confidence Interval (%)
AML1, Red	21q22.1	200	200	100	98.12 - 100
ETO, Green	8q21.3	200	200	100	98.12 - 100

Analytical Sensitivity
Analytical sensitivity is defined as the percentage of scoreable interphase cells with the expected normal signal pattern.
The analytical sensitivity of the AML1/ETO (RUNX1/RUNX1T1) Translocation Dual Fusion FISH Probe Kit, was established by analyzing interphase nuclei from 25 bone marrow samples, selected from the intended population for the probe. Each sample was analyzed by two independent analysts and the signal pattern of each interphase was recorded. Each analyst analyzed 100 nuclei per sample, for a total of 200 nuclei per sample, resulting in 5000 scorable nuclei per probe evaluated. The sensitivity was calculated as the percentage of scoreable cells with the expected signal pattern and is given below with a 95% confidence interval.

Table 2. Analytical Sensitivity for the CytoCell AML1/ETO (RUNX1/RUNX1T1) Translocation, Dual Fusion FISH Probe Kit

Probe	Number of interphase nuclei with the expected normal signal pattern	Total number of interphase nuclei analyzed	Analytical sensitivity (%)	95% Confidence interval (%)
USA-LPH026 AML1/ETO (RUNX1/RUNX1T1) Translocation, Dual Fusion Probe	4965	5000	99.30	99.03 - 99.5

Characterization of Normal Cut-off Values
The limit of detection of a FISH test is established by calculating the upper limit of the abnormal signal pattern in normal cells. This upper limit constitutes the "normal cut-off value". The normal cut-off value, 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.
An analytical result above the normal cut-off (upper reference limit) is deemed to have detected the rearrangement. Cut-off values are given for each expected abnormal signal pattern. Conversely, an analytical result below the cut-off is deemed to be negative for the rearrangement.
The normal cut-off was calculated using the Microsoft Excel β inverse function (BETAINV). Beta inverse uses the upper bound of a one-sided 95% confidence interval of the binomial distribution rearrangement.
Laboratories must verify cut-off values using their own data.
The normal cut-off for the AML1/ETO (RUNX1/RUNX1T1) Translocation Dual Fusion FISH Probe Kit, was established, using the data from 1290 bone marrow samples negative for the rearrangement the probe is intended to detect. Two independent analysts analyzed a target number of 100 interphase nuclei per sample.
The normal cut-off value for the '1R, 1G, 2F' signal pattern when using the AML1/ETO (RUNX1/RUNX1T1) Translocation Dual Fusion FISH Probe Kit was calculated to be 2.3% using the beta inverse function.

Table 3. Characterization of Normal Cut-off Values for the CytoCell AML1/ETO (RUNX1/RUNX1T1) Translocation, Dual Fusion FISH Probe Kit

Abnormal signal pattern	Number of samples analyzed to generate the cut-off	Number of nuclei evaluated per sample	Maximum number of false positive signal pattern	Normal cut-off value (per 200 nuclei)	Normal cut-off value (%)
1R, 1G, 2F	1290	200	1	5	2.3

Reproducibility
Reproducibility is a measure of the variability of a test and has been established in terms of site-to-site, day-to-day and batch-to-batch variability. Site-to-site and day-to-day reproducibility were assessed by analyzing two replicates of two high-positive, two near-cut-off/low-positive and two normal bone marrow samples on each of five days at three sites. For each sample, a total of 200 interphase cells was scored by two analysts and the probe signal patterns recorded. The percentage of cells with the expected signal pattern was calculated. Lot-to-lot reproducibility, was assessed by analyzing four replicates of one high-positive, one low-positive and one normal bone marrow sample on three different batches of probe at one site. A total of 200 interphase nuclei were analyzed per spot, by two independent analysts and the probe signal patterns recorded. The results of the data were analyzed and the overall, negative and positive percent agreement for each sample summarized. The agreement percentages for the High Positive and Negative samples were 100% and 100% respectively.

Table 4. Specimen Agreement Site-to-Site Reproducibility for the CytoCell AML1/ETO (RUNX1/RUNX1T1) Translocation, Dual Fusion FISH Probe Kit

Category	Agree	Disagree	Total	Agreement (%)
Negative 1	30	0	30	100
Negative 1	30	0	30	100
Near cut-off 1	24	6	30	80
Near cut-off 2	29	1	30	97
Positive 1	30	0	30	100
Positive 2	30	0	30	100

Table 5. Site-to-Site Analysis of Variance – Standard Deviation (SD) for the CytoCell AML1/ETO (RUNX1/RUNX1T1) Translocation, Dual Fusion FISH Probe Kit

Specimen	N	Mean of abnormal percentage	Intra-Day SD	Inter-Day SD	Between-site SD	Total SD
Negative 1	30	0.00	0.00	0.00	0.00	0.00
Negative 1	30	0.00	0.00	0.00	0.00	0.00
Near cut-off 1	30	3.92	0.23	0.00	0.12	1.72
Near cut-off 2	30	5.65	1.57	0.00	0.82	2.40
Positive 1	30	74.28	0.00	1.06	0.00	8.30
Positive 2	30	82.05	0.25	0.00	0.00	6.25

Clinical Study
The AML1/ETO (RUNX1/RUNX1T1) Translocation, Dual Fusion FISH Probe Kit was used by two different clinical laboratories on unselected AML/MDS samples. The number of abnormal samples identified showing the expected abnormal signal pattern was compared with the expected incidence rate from a literature source review. The results fell within the expected range derived from the literature review.

Cited published literature may discuss device uses that have not been approved or cleared by FDA.

Table 6a. Clinical Study Results for the CytoCell AML1/ETO (RUNX1/RUNX1T1) Translocation, Dual Fusion FISH Probe Kit – Confirmed AML Cases

Condition	Literature Source 1 Papaemmanuil et al.	Literature Source 2 Grimwade et al.	Literature Source 3 Dores et al.
Was the specific device under review in the submission used in the study?	No	No	No
Was the specimen type in the study representative of the claimed specimen type(s)	Yes	Yes	Yes
Target population (disease status)	Confirmed AML	Confirmed AML	Confirmed AML
Upper reference limit - 'Cut-off value' (percentage and per 200 nuclei)	N/A	N/A	N/A
Total Number of specimens tested for each claimed type	1540	5876	19497
Number of specimens with a positive probe result	N/A	N/A	N/A
Range of positive probe results	N/A	N/A	N/A
Source incidence rate for rearrangement %	3.8%	7%	1.6%

Table 6b. Results from Clinical Laboratory Testing using the CytoCell AML1/ETO (RUNX1/RUNX1T1) Translocation, Dual Fusion FISH Probe Kit

Condition	Data Source 1	Data Source 2
Was the specific device under review in the submission used in the study?	Yes	Yes
Was the specimen type in the study representative of the claimed specimen type(s)	Yes	Yes
Target population (disease status)	Known or suspected MDS or AML	Known or suspected MDS or AML
Upper reference limit - 'Cut-off value' (percentage and per 200 nuclei)	2.3% or 5 1R1G2F patterns per 200 scoreable interphase nuclei	2.3% or 5 1R1G2F patterns per 200 scoreable interphase nuclei
Total Number of specimens tested for each claimed type	100	414
Number of specimens with a positive probe result	0	6
Range of positive probe results	N/A	69.5%-98.5%
Source incidence rate for rearrangement % (95% CI)	0% (0.00% to 3.62%)	1.45% (0.53% to 3.13%)
Expected Range from Literature	1.6% - 7.0%	

Additional Information
For additional product information please contact the CytoCell Technical Support Department.
T: +44 (0)1223 294048
E: techsupport@cytocell.com
W: www.ogt.com

References

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REF	**EN:** Catalogue number
IVD	**EN:** *In vitro* diagnostic device
LOT	**EN**: Batch code
	EN: Consult instructions for use
	EN: Manufacturer
	EN: Use by
	EN: Temperature limitation
Σ	**EN:** Sufficient for <n> tests
CONT	**EN:** Contents
Rx only	**EN:** For prescription use only

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