A Sysmex Group Company

CytoCell

Instructions For Use

REF: LPH 067-S / LPH 067

CLL *PROFILER* Kit

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 genomic losses larger than the region covered by the red and green clones in this probe set which include the P53 (TP53), ATM and D13S319 regions or gains larger than the region covered by the blue clone in this probe set, which includes the chromosome 12 centromere. Genomic gains/losses outside these regions or partial gains/losses of this regions 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 CLL *PROFILER* Kit is a qualitative, non-automated, fluorescence *in situ* hybridisation (FISH) test used to detect chromosomal deletions in the 11q22.3 region on chromosome 11, the 17p13.1 region on chromosome 17 or the 13q14.2-q14.3 region on chromosome 13 and/or gains of the centromeric region on chromosome 12 in Carnoy's solution (3:1 methanol/acetic acid) fixed haematologically-derived cell suspensions from patients with chronic lymphocytic leukaemia (CLL).

Indications

This product is designed as an adjunct to other clinical and histopathological tests in recognised diagnostic and clinical care pathways, where knowledge of *P53 (TP53), ATM* deletion or D13S319 deletion status and/or gain of chromosome 12 centromere 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

The CytoCell CLL PROFILER Kit is intended to detect deletions of TP53, ATM and D13S319, and gains of the chromosome 12 centromere sequences in peripheral blood or bone marrow samples from patients with chronic lymphocytic leukaemia (CLL).

D13S319/13qter/12cen Deletion/Enumeration

Deletions affecting 13q14 are also the most frequent structural genetic aberrations in chronic lymphocytic leukaemia (CLL)[6,7,8]. This region is found to be heterozygously deleted in 30-60% and homozygously deleted in 10-20% of CLL patients[9]. The survival rate has been shown to be similar for the two groups[10]. Patients with 13q14 deletions are classified as very low risk, in the absence of any other genetic lesions[1].

Two non-coding RNA genes, DLEU1 (*deleted in lymphocytic leukemia 1*) and DLEU2 (*deleted in lymphocytic leukemia 2*), plus the genetic marker D13S319, span the pathogenic critical region of 13q1411. DLEU1 is considered to be the most likely CLL-associated candidate tumour suppressor gene within the 13q14 region[12]. Trisomy 12 is a recurrent abnormality in CLL, seen in 20% of the cases[13] and often appears as the unique cytogenetic aberration (40-60% of cases with trisomy 12)[7]. Patients with trisomy 12 are classified as low-risk in the absence of any other genetic lesions[1].

P53(TP53)/ATM Probe Combination

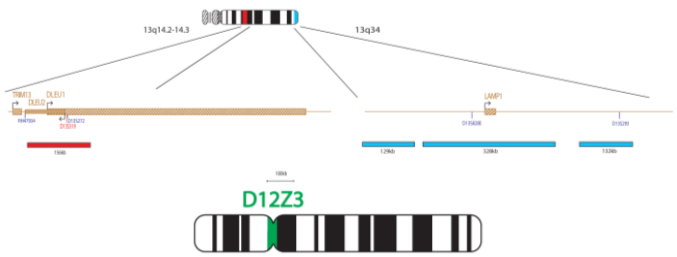
The TP53 (*tumor protein p53*) gene at 17p13.1 is one of most important tumour suppressor genes; it acts as a potent transcription factor with fundamental role in the maintenance of genetic stability. Loss of TP53 is reported in 10% of patients with CLL, and is considered to be the poorest prognostic marker in that disease[1,2].

The ATM (*ATM serine/threonine kinase*) gene at 11q22.3 is an important checkpoint gene involved in the management of cell damage; its function is to assess the level of DNA damage in the cell and attempt repair by phosphorylating key substrates involved in the DNA damage response pathway[3]. Loss of ATM is reported in 18% of patients with CLL, and is considered a poor prognostic marker in that disease[4].

Analysis of the ATM/TP53 interaction in CLL has shown that TP53 and ATM play an important role in the proliferation of lymphoid cancer[3]. It has been shown that ATM enhances the phosphorylation of TP53, should the damage be so great that the cell requires destruction by apoptosis (which is mediated by TP53). Deletion of ATM removes this checkpoint activity and hence activation of TP53. Thus, there is no attempt made to repair, or apoptosis of, damaged cells, despite the presence of TP53. In the absence of ATM, damaged cells are allowed to continue to proliferate[5].

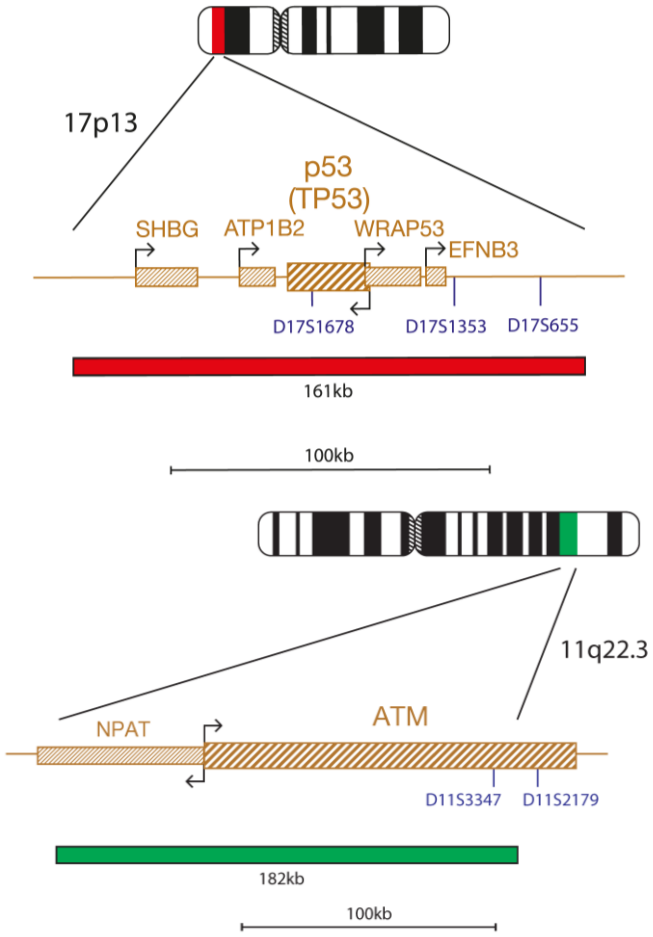
Probe Specification

D13S319/13qter/12cen Deletion, Enumeration Probe
D13S319, 13q14.2, Red
13qter, 13q34, Blue
D12Z3, 12p11.1-q11.1, Green



The Chromosome 12 Alpha Satellite Probe is a repeat sequence probe, labelled in green, which recognises the centromeric repeat sequence D12Z3. The D13S319 probe, labelled in red, covers a 156kb region including the entire DLEU1 and most of the DLEU2 genes and the D13S319, D13S272 and RH47934 markers. The 13qter subtelomere specific probe, labelled in blue, allows identification of chromosome 13 and acts as a control probe.

P53 (TP53)/ATM
P53, 17p13.1, Red
ATM, 11q22.3, Green



The P53 component consists of a 161kb probe, labelled in red that covers the whole P53 (TP53) gene and flanking regions. The ATM component consists of a 182kb probe, labelled in green that covers the telomeric end of the NPAT gene and the centromeric end of the ATM gene beyond the D11S3347 marker.

Materials Provided
D13S319/13qter/12cen Deletion, Enumeration Probe:
50µl per vial (5 tests) or 100µl per vial (10 tests)

P53 (TP53) /ATM 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]
Aqua	418	467
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. Use a single bandpass aqua spectrum filter for optimal visualisation of the aqua spectrum or a triple bandpass red spectrum/green spectrum/aqua spectrum filter for simultaneous visualisation of the green, red and aqua 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 peripheral blood cells or 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[14].

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.

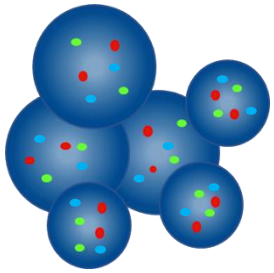
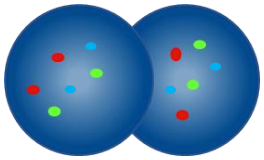
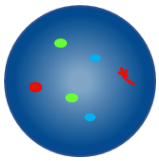
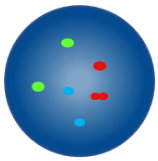
Post-Hybridisation Washes
12. Remove the DAPI from the freezer and allow it to warm to RT.
13. Remove the coverslip and all traces of glue carefully.
14. Immerse the slide in 0.4xSSC (pH 7.0) at 72ºC (+/- 1ºC) for 2 minutes without agitation.
15. Drain the slide and immerse it in 2xSSC, 0.05% Tween-20 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

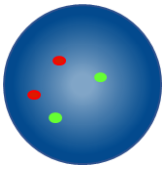
Interpretation of Results
Assessing Slide Quality
The slide should not be analysed if:
- Signals are too weak to analyse in single filters - in order to proceed with analysis, signals should appear bright, distinct and easily evaluable
- There are high numbers of clumped/overlapping cells obstructing 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, two blue signals and two green signals – one of the two red signals is diffuse
	Count as two red signals, two blue signals and two green signals – the gap in one red signal is less than two signal widths

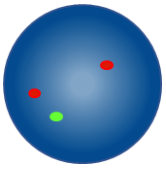
Expected Results

P53/ATM Probe
Expected Normal Signal Pattern

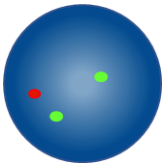


In a normal cell, two red and two green signals (2R, 2G) are expected.

Expected Abnormal Signal Patterns



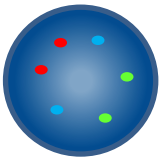
In a cell with an ATM deletion, the expected signal pattern will be two red and one green signal (2R, 1G).



In a cell with a P53 deletion, the expected signal pattern will be one red and two green signals (1R, 2G).

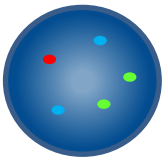
D13S319/13qter/12cen Deletion, Enumeration Probe

Expected Normal Signal Pattern

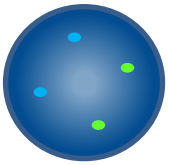


In a normal cell, two red, two blue and two green signals (2R, 2B, 2G) are expected.

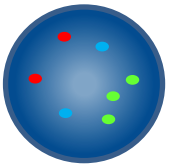
Expected Abnormal Signal Patterns



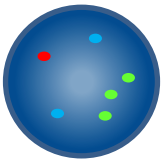
In a cell with a hemizygous deletion of the D13S319 locus, the expected signal pattern will be one red, two blue and 2 green signals (1R, 2B, 2G).



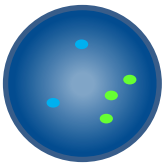
In a cell with a homozygous deletion of the D13S319 locus, the expected signal pattern will be zero red, two blue and two green signals (0R, 2B, 2G).



In a cell with trisomy 12 and normal D13S319 status, the expected signal pattern will be two red, two blue and three green signals (2R, 2B, 3G).



In a cell with trisomy 12 and a hemizygous D13S319 deletion, the expected signal pattern will be one red, two blue and three green signals (1R, 2B, 3G).



In a cell with trisomy 12 and a homozygous D13S319 deletion, the expected signal pattern will be zero red, two blue and three green signals (0R, 2B, 3G).

Other signal patterns are possible in aneuploid/unbalanced specimens.

Known Cross-Reactivity

The green D12Z3 probe may show cross hybridisation to 3c, 6c, 7c and 10c.

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.

Table 1. Analytical Specificity for the CLL *PROFILER* Kit

Kit	Probe	Target Locus	No. of Signals Hybridised to the Correct Locus	Total No. of Signals Hybridised	Specificity (%)
D13S319/ 13qter/12cen Deletion, Enumeration Probe	Red D13S319	13q14.2	200	200	100
	Blue 13qter	13q34	200	200	100
	Green D12Z3	12p11.1-q11.1	200	200	100
P53/ATM Probe	Red P53	17p13	200	200	100
	Green ATM	11q22.3	200	200	100

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).

Table 2. Analytical Sensitivity for the CLL *PROFILER* Kit

Kit	No. of Cells with Expected Signal Patterns	No. of Cells with Scoreable Signals	Sensitivity (%)	95% Confidence Interval
D13S319/ 13qter/12cen Deletion, Enumeration Probe	467	500	93.4	2.6
P53/ATM Probe	479	500	95.8	1.7

Characterisation of Normal Cut-off Values

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.

The normal cut-off value was established using samples from normal and positive patients. 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.

Table 3. Characterisation of Normal Cut-off Values for the CLL *PROFILER* Kit

Kit	Rearrangement	Abnormal signal pattern	Youden Index	Normal Cut-off (%)
D13S319/ 13qter/12cen Deletion, Enumeration Probe	D13S319 hemizygous deletion	1R, 2B, 2G	0.96	6
	Trisomy 12	2R, 2B, 3G	0.99	4
P53/ATM Probe	P53 deletion	1R, 2G	0.99	8
	ATM deletion	2R, 1G	0.99	8

Laboratories must verify cut-off values using their own data[15,16].

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 same lot number of probe tested on the same sample, in the same conditions on the same day.

Reproducibility is a measure of the 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.

Table 4. Reproducibility and Precision for the CLL *PROFILER* Kit

Variable	Standard Deviation (STDEV)	
	D13S319/13qter/12cen Deletion, Enumeration Probe	*P53/ATM Probe*
Precision	1.28	1.37
Sample-to-sample	1.30	1.60
Day-to-day	4.12	2.27
Batch-to-batch	2.04	1.77
Overall deviation	3.30	1.98

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 5. Clinical Performance for the CLL *PROFILER* Kit

Rearrangement	Clinical Sensitivity (true positive rate, TPR)	Clinical Specificity (true negative rate, TNR)	False Positive rate (FPR) = 1 – Specificity
D13S319/13qter/12cen Deletion, Enumeration Probe			
D13S319 Deletion	96.6%	99.5%	0.5%
Trisomy 12	100%	100.0%	0%
P53/ATM Probe			
P53 deletion	100%	100%	0%
ATM deletion	100%	100%	0%

Additional Information

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

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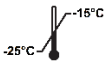
Guide to Symbols

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
Σ	**en:** Contains sufficient for <n> tests
CONT	**en:** Contents

Patents and Trademarks

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