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For Professsional Use Only

AML1/ETO gene fusion detection kit Instructions Manual

CE IVD

[Product Name] AML1/ETO gene fusion detection kit

[Package Specifications] 10 Tests/box

[Intended use]

This product is suitable for qualitative detection of AML1/ETO fusion gene in bone marrow samples of leukemia patients.

It is only used for the auxiliary diagnosis of molecular typing of treated patients.

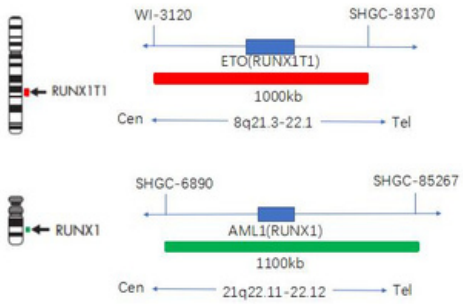
Leukemia is a kind of malignant clonal disease of hematopoietic stem cells. Clonal leukemia cells proliferate and accumulate in bone marrow and other hematopoietic tissues, infiltrate other non hematopoietic tissues and organs, and inhibit normal hematopoietic function. AML1/ETO fusion gene is a common cytogenetic abnormality in patients with acute myeloid leukemia (AML). About 12%~20% of AML patients have AML1/ETO fusion gene, while the positive rate in AML-M2 leukemia is 20%~40%, of which the positive rate in M2b subtype is as high as 90%, which is rare in other subtypes. Patients with simple AML1/ETO fusion gene positive have better prognosis and high complete remission rate.

This kit is only applicable to the detection of AML1/ETO fusion gene status. The test results are only for clinical reference and should not be used as the only basis for patient diagnosis and treatment. Clinicians should comprehensively judge the test results in combination with other clinical test indicators and other factors.

[Detection principle]

Fluorescence in situ hybridization is a technique for directly observing specific nucleic acids in cells in vitro. According to the principle of base complementary pairing, the specific probe is complementary to the target sequence in the cell. Due to the fluorescence of the probe, the gene state of the hybrid probe and the target sequence can be clearly observed under the fluorescence microscope under the appropriate excitation light.

The kit uses orange fluorescein to label ETO probe and green fluorescein to label AML1 probe. The two probes can be combined with the target detection site by in situ hybridization. Under normal conditions (AML1/ETO gene does not fuse), it is displayed as two orange signals and two green signals under fluorescence microscope. When there is a fusion gene, the green and orange signals form a yellow fusion signal due to recombination.



[Product Composition]

The kit consists of AML1/ETO dual-color probes, as shown in Table 1.The reagents not provided in the kit are shown in Table 2.

Table 1 Kit composition

Component name	Specifications	Quantity	Main components
AML1/ETO dual color probe	100μl/Tube	1	ETO Orange probe,AML1 Green probe

Table 2 List of reagents not provided

Reagent name	Purity	Reagentname	Purity
Sodium chloride	Analytical purity AR	NP-40	Analytical purity AR
Sodium citrate	Analytical purity AR	Xylene	Analytical purity AR
Anhydrous ethanol	Analytical purity AR	Protease K	≥40 units/g

[Storage conditions & Validity]

Keep sealed away from light at -20℃±5℃. The product is valid for 12 months. Avoid unnecessary repeated freezing and thawing that should not exceed 10 times. After opening, within 24 hours for short-term preservation, keep sealed at 2~8℃ in dark. For long-term preservation after opening, keep the lid sealed at -20℃±5℃ away from light. See the label of the kit for the production date and expiration date.

[Applicable Instruments]

1. Fluorescence microscopy imaging system includes fluorescence microscope and filter sets. The kit is labeled with orange fluorescein, and the

filter set compatible with the fluorescent labeled dye should be selected.

Orange fluorescence: The maximum excitation wavelength is 552nm and the maximum emission wavelength is 576nm.

Green fluorescence: The maximum excitation wavelength is 496nm and the maximum emission wavelength is 520nm.

Fluorescence microscopy imaging system should use a microscope with orange and green channels. For monochromatic channel microscope, image synthesis analysis results should be used.

2. Automatic hybridization instrument: Strict temperature uniformity is required, and the temperature difference should be ≤1℃.

[Sample Requirements]

1. Applicable specimen type: unfixed fresh bone marrow specimen (stored at 2~8 ℃ for no more than 24 hours).

2. Sample collection: 1~3ml heparin sodium anticoagulant

3. Sample preservation: after fixation, the cell suspension shall be stored at -20±5℃ for no more than 12 months, and the freezing and thawing times of the sample shall not exceed 6 times; The prepared cell slides can be stored at -20±5℃ for no more than 1 month. When the storage temperature of the sample is too high or too low (such as freezing), and the cell suspension is over volatilized or polluted during storage, the sample will not be used for detection

[Test method]

1.Related Reagents

The following reagents are required for the experiment but not provided in this kit

① **20×SSC, pH 5.3±0.2**

Weigh 176g of sodium chloride and 88g of sodium citrate, dissolve in 800mL of deionized water, adjust the pH to 5.3±0.2 at room temperature, and complete to 1 L with deionized water. High-pressure steam sterilization, stored at 2-8℃, the solution shelf life is of 6 months. Discard if the reagent appears cloudy (turbid) or contaminated.

Sodiumchloride	176g
Sodiumcitrate	88g

② **2×SSC, pH 7.0±0.2**

Take 100mL of the above 20xSSC, dilute with 800mL deionized water, mix, adjust the pH to 7.0±0.2 at room temperature, complete to 1L with deionized water, stored at 2-8℃, the shelf life is of 6 months. Discard if the reagent appears cloudy (turbid) or contaminated.

③ **Ethanol Solution: 70% ethanol, 85% ethanol**

Dilute 700ml, 850ml of ethanol with deionized water to 1L. The shelf life is of 6 months. Discard if the reagent appears cloudy (turbid) or contaminated.

④ **0.3% NP-40/0.4xSSC solution, pH 7.0-7.5**

NP-40	0.6mL
20xSSC	4mL

Take 0.6mL NP-40 and 4mL 20×SSC, add 150mL deionized water, mix, adjust the pH to 7.0-7.5 at room temperature, with deionized water complete to a volume of 200mL. Stored at 2-8℃, the shelf life is of 6 months. Discard if the reagent appears cloudy (turbid) or contaminated.

⑤**Fixation solution (methanol: glacial acetic acid = 3:1)**

Prepare a ready to use fixation solution by mixing thoroughly 30ml of methanol and 10ml of glacial acetic acid.

⑥ **0.075M KCl solution**

Weigh 2.8g of potassium chloride, dissolve in 400mL of deionized water and complete to 500mL with deionized water. Stored at room temperature, the solution shelf life is of 6 months. Discard if the reagent appears cloudy (turbid) or contaminated.

⑦ **Diamidinyl phenylindole (DAPI) counterstain**

Use commercially available anti-quenching DAPI counterstain.

2. Sample collection and slides preparation

① Sample collection: Take anticoagulated bone marrow cell samples.

② Cell harvest: The bone marrow cells sample is pipetted to the tip of centrifuge tube and centrifuge at 1000rpm for 10 minutes to remove the supernatant.

③ Cells hypotonicty: Add 6-8mL of 0.075mol/L KCl solution pre-warmed to 37℃, mix with a pipette and place in an incubator at 37℃ for 20-30min.

④ Pre-fixation: Add 2 mL of 3:1 methanol and glacial acetic acid fixative solution, mix with a pipette, and centrifuge at 1000 rpm for 10 min.

⑤ Fixation: Aspirate the supernatant, add 5 mL of freshly prepared 3:1 methanol and glacial acetic acid fixative solution, mix with a pipette, fix

for 10 min, and centrifuge at 1000 rpm for 10 min.

⑥ Repeat step 5 until the cell pellet is washed and cleaned.

⑦ Cell suspension preparation: Pipet the supernatant and add the appropriate amount of fixative solution to prepare the appropriate cell suspension concentration.

⑧ Slide preparation: absorb 3 ~ 5μL Drop the L cell suspension onto the anti detachment slide (it is recommended that the cell density of the sample drop is 5×10^3~5×10^4 cells/μL) , aging at 56 ℃ for 0.5~2 hours.

⑨ The prepared slides can be stored at -20±5 ℃ for no more than 4 weeks.

3. Slides preparation

① At room temperature with 2×SSC (pH 7.0) solution, rinse the slide 2 times for 5min each time.

② Place the slides in 70% ethanol, 85% ethanol and 100% ethanol for 2min each time, dehydrate and air dry.

4. Denaturation and Hybridization

The following operations should be performed in a darkroom.

① Take the probe at room temperature for 5 minutes. Briefly centrifuge manually (do not use vortex or shaker instrument). Take 10μl droplet in the cell and drop in the hybridization zone, immediately cover 22mmx22mm glass slide area; spread evenly without bubbles the probe under the glass slide covered area and seal edges with rubber (edge sealing must be thorough to prevent dry film from affecting the test results during hybridization).

② Place the glass slide in the hybridization instrument, denature at 88°C for 2 minutes (the hybridizer should be preheated to 88ºC) and hybridize at 45°C for 2 to 16 hours.

5. Washing

The following operations should be performed in a darkroom.

① Take out the hybridized glass slides, remove the rubber on the coverslip and immediately place the slides into 2xSSC for 5 seconds, and gently remove the coverslip.

② Place the glass slides in 2xSSC at room temperature for 1 min.

③ Remove and immerse the slides in a 0.3% NP-40/0.4×SSC solution preheated at 68°C for 2 min. (Preparation of 0.3% NP-40/0.4xSSC: For 1L preparation, take 3mL NP-40 and 20mL 20xSSC, dissolve fully, mix well, and use 1M NaOH to adjust the pH to 7.2).

④ Immerse the glass slides in deionized water at 37ºC for 1min, and dry naturally in the dark.

6. Counterstaining

The following operations should be performed in a darkroom

10μl DAPI compound dye is dropped in the hybridization area of the glass slide and immediately covered. The suitable filter is selected for glass slide observation under the fluorescence microscope.

7. Preservation of slides after hybridization

After hybridization, washing and re staining, the slides can be sealed and stored in the dark at -20℃±5℃, and can be observed under normal microscope within 20 days.

8. FISH results observation

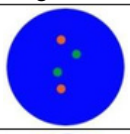
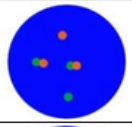
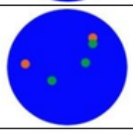
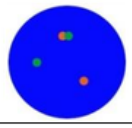
① Results observation method: put the counterstained glass slide under the fluorescence microscope, and first put it under the low power objectivelens(10×） toconfirmthecellareaunderthemicroscope;Goto40×Undertheobjectivelens,findapositionwherethecellsareevenly distributed; Then in the high-power objective (100×), The FISH results of nuclei were observed. During microscopic examination, the continuous irradiation time of a single visual field under the green channel and red channel shall be controlled within 40 minutes.

② Interpretable sample standard: the hybridization signal of the probe is bright and clear, the orange and green signals are easy to distinguish, the spontaneous fluorescence does not affect the signal count, and the number of countable cells is not less than 200.

③ Countable cell standard: the cell distribution is reasonable, there is no overlap, DAPI counterstaining is clear, that is, the nuclear boundary is clear, and the number of orange, green signals or yellow signals formed by fusion in cells is ≥ 1.

④ Counting method: randomly count 200 cells in each sample, count the number of orange, green and yellow fusion signals in each nucleus, and calculate the ratio of cells showing abnormal cell signal mode (number of abnormal cells / number of counted cells) × 100%）。

Place the stained sections under a fluorescence microscope and the cells area is first confirmed under a low magnification objective (10×); under magnification objective (40×) a uniform cells distribution is observed; then the nucleus size uniformity, nuclear boundary integrity, DAPI staining uniformity, no nuclei overlapping, cells clear signal are observed in the high magnification objective (60x, 100x). Select randomly 200 cells at least and count the orange and green signals in the nuclei.

Signal type	Diagram pattern ● orange signal ● green signal	Cells results determination
2 orange red signals, 2 green signals (2R2G)		Negative
1 orange signal, 1 green signal, 2 fusion signals (1R1G2F)		Positive
1 orange signal, 2 green signals, 1 fusion signal (1R2G1F)		Positive
1 orange signal, 1 green signal, 1 fusion signal (1R1G1F)		Positive

2. Threshold setting

Each laboratory should set the threshold independently: randomly select 20 human bone marrow blood cell samples, process them according to the sample processing requirements, and prepare abnormal threshold reference tablets. 200 cells were randomly counted for each reference slice, the number and percentage of cells with various types of positive cells were calculated, and the average value and standard deviation of the percentage were counted.

① Calculate the percentage of the total number of cells with 1r1g1f signal type, the average value and standard deviation of the statistical percentage, and set the abnormal threshold as the average value + 3 times the standard deviation, which is recorded as threshold a.

② Calculate the percentage of the total number of cells with other fusion signal types except 1r1g1f, and count the average value and standard deviation of the percentage. The abnormal threshold is set as the average value + 3 times the standard deviation, which is recorded as threshold B.

The threshold a established by the company is 11.5% and the threshold b is 3.0%, which can be used as a reference. Due to the difference of sample processing methods and the subjectivity of signal counting, the threshold of each laboratory will be different. Each laboratory should establish the threshold in strict accordance with the standard process of threshold setting.

[Interpretation of test results]

1. Result judgment

After counting 200 cells, the number and percentage of various types of positive cells were calculated respectively,

① If the ratio of positive cells of 1r1g1f signal type > threshold a, it is judged as positive;

② Except for 1r1g1f signal type, the ratio of positive cells of other fusion signal types > threshold b, which is judged to be positive.

③ If the ratio of positive cells of 1r1g1f signal type is less than threshold a, it is judged as negative.

④ Except for 1r1g1f signal type, the ratio of positive cells of other fusion signal types < threshold b, which is interpreted as negative.

⑤ If the ratio of positive cells = threshold (a or b), increase the number of counts or analyze the whole film. If it is still less than or equal to the abnormal threshold, it will be interpreted as negative, otherwise it will be interpreted as positive.

⑥ If the ratio of positive cells is within ± 3 times the standard deviation of the mean value, the sample should be treated with caution.

2. Judgment of invalid experiment

① If the number of cells available for probe analysis is less than 200, this test shall be judged as invalid.

② If the fluorescence hybridization signal intensity or background available for analysis is not ideal or clear, which affects the judgment of results, this detection shall be judged as unreliable and treated as invalid experiment.

3. Common problems and treatment methods in the experiment

The factors affecting the test results and treatment methods in the experiment are shown in Table 4:

Table 4 FAQ and solutions

Question	Possible cause	Recommended solution

Question	Possible cause	Recommended solution
background is too strong	Slides were not cleaned properly before specimen's preparation.	Follow the recommended procedures for washing glass slides.
	Inadequate washing after hybridization.	Ensure that the washing solution is prepared according to the instructions, ensure the correct pH value and temperature of the washing solution, remove the coverslip and repeat the washing steps.
	Improper use of filter sets.	Replace the appropriate filter sets for observation and to weaken the background light.
	Improper hybridization conditions.	Ensure that the hybridization instrument temperature is 45°C.
	Low washing temperature.	Ensure that the solution temperature is at the washing slides required temperature.
Too weak dye	Too weak dye.	68°C 0.3%NP-40/0.4 × In SSc solution, shake for 10 ~ 20 seconds, remove the cover glass and soak for 2 minutes. Place the slide in deionized water at 37 °C and soak it for 1 minute. Dry the slide naturally in the dark and then re dye it.
	Obsolete dye agent or excessive illumination	Ensure that the dye agent is stored at -20°C and keep away from light. Make sure that the dye agent is valid.
No signal or weak signal	Specimen incomplete denaturation.	Ensure that the hybridization instrument temperature is at 88°C, and the hybridization instrument should be preheated at least 10min ahead of time.
	Improper pre-denaturation specimens' preparation.	Please refer to the above sample preparation related questions and answers.
	Probes and hybridization buffer improper mixture before usage.	Mix well the probe and the hybridization buffer, centrifuge briefly.
	Probe mixture on the slide dries too fast	Wash the coverslip in the washing solution.
	Bubbles formation under coverslips during hybridization.	When covering the coverslip, cover the surface of the probe mix and squeeze gently to allow the bubbles to escape.
	Inappropriate hybridization conditions.	Ensure to observe time and temperature specified for the hybridization; do not leave gaps in the rubber seals; adjust hybridization time as appropriate.
	Improper washing solution or washing conditions.	Ensure that the washing solution is prepared according to the product specification; Check that the washing solution temperature reaches the in the washing step specified temperature; Assure that the thermometer and the pH meter are correctly calibrated.
	Probe or specimen slides inadequate storage.	Ensure that the probe is stored in dark at -20°C. Place the unhybridized slides dry at -20°C for a long conservation or at room temperature for a short storage. Place the hybridized slides at -20°C and store in dark. The storage period should not exceed 6 months.
	Incorrect dye agent or too bright dye agent usage.	68°C 0.3%NP-40/0.4 × In SSc solution, shake for 10 ~ 20 seconds, remove the cover glass and soak for 2 minutes. Place the slide in deionized water at 37 °C and soak it for 1 minute. Dry the slide naturally in the dark and then re dye it.
	The selected filter sets is unsuitable for observation.	Use the correct filter sets to observe the probe fluorescence. For details, please contact Wuhan HealthCare Biotechnology Co., Ltd. Technical Service Department.

[Limitations of test methods]

This kit is only used for the detection of AML1/ETO fusion gene, and cannot detect the fusion state of AML1 gene and other genes. This kit is only applicable to the detection of AML1/ETO fusion gene status, and the test results are only for clinical reference.

[Product performance index]

1. Appearance: the outer package of the kit is complete without damage, and the marks are complete and clear; The liquid reagent shall be clearly marked without leakage.

2. Fluorescence signal intensity: after the probe is effectively hybridized with the karyotype reference, it will send out fluorescence signals that can be recognized by the naked eye under the fluorescence microscope.

3. Sensitivity: after the probe effectively hybridized with the karyotype reference, 100 chromosomes 21 of 50 cells in metaphase were analyzed, and at least 98 chromosomes 21 showed 1 green fluorescence signal; 100 chromosomes 8 of 50 cells in metaphase were analyzed, and at least 98 chromosomes 8 showed an orange fluorescence signal.

4. Specificity: after the probe effectively hybridized with the karyotype reference, 100 chromosomes 21 of 50 cells in metaphase were analyzed, and at least 98 chromosomes 21 showed a specific green fluorescence signal in the long arm target region; 100 chromosomes 8 of 50 cells in metaphase were analyzed, and at least 98 chromosomes 8 showed a specific orange fluorescence signal in the long arm target region.

5. Coincidence rate of negative and positive: five negative reference materials (bone marrow cells of leukemia patients with clinical diagnosis of AML1/ETO fusion gene negative) were detected, and the fluorescence signals were analyzed. The results met the negative judgment criteria and were judged to be negative. Five positive reference materials (bone marrow cells of leukemia patients clinically diagnosed as AML1 / ETO fusion gene positive) were detected and the fluorescence signals were analyzed. The results met the positive judgment criteria and were all judged to be positive.

6. Precision: the precision of this kit within batch, between batches, within operation, between operation, during the day, and between operators / film readers is less than 5%.

7. Interfering substance: heparin sodium as anticoagulant will not interfere with the test results.

8. Clinical trial: among the 200 samples tested by this kit and the comparison reagent at the same time, the positive coincidence rate, negative

coincidence rate and overall coincidence rate of this kit were 100%, and the kappa value was 1.00 ($P < 0.001$); In 1086 samples detected simultaneously by this kit and karyotype analysis, the positive coincidence rate of this kit was 98.28%, the negative coincidence rate was 100%, the overall coincidence rate was 99.91%, and the kappa value was 0.99 ($P < 0.001$).

[Precautions]

1. Please read this manual carefully before testing. The testing personnel shall receive professional technical training, and the signal counting personnel must be able to observe and distinguish orange and green signals. The use of matching reagents not described in the instructions or standard operation not in accordance with the instructions may affect the test results.
2. When testing clinical samples, when it is difficult to count the hybridization signal and the samples are not enough to repeat the retest, the test will not provide any test results. If the amount of cells is insufficient for analysis, the test will not provide test results.
3. Formamide and DAPI counterstaining agent used in this experiment have potential toxicity or carcinogenicity. It is necessary to operate in the fume hood and wear masks and gloves to avoid direct contact.
4. The results of this kit will be affected by various factors of the sample itself, but also limited by hybridization temperature and time, operating environment and the limitations of current molecular biology technology, which may lead to wrong AML1/ETO fracture fusion gene results. Users must understand the potential errors and accuracy limitations that may exist in the detection process.
5. All chemicals are potentially dangerous. Avoid direct contact. Used kits are clinical waste and should be properly disposed of.

[References]

[1].ZHOU G B,KANG H,WANG L,et al.Oridonin, a diterpenoid extracted from medicinal herbs, targets AML1-ETO fusion protein and shows potent antitumor activity with low adverse effects on t (801)leukemia in vitro and vivo[J].Blood, 2007,109:3441-3450.
[2].NCCN Clinical Practice Guidelines in Oncology –Acute Myeloid leukemia (Version 1.2015).
[3]. Society of Hematology, Chinese Medical Association Chinese guidelines for diagnosis and treatment of acute myeloid leukemia (recurrent and refractory) (2011 Edition) [J] Chinese Journal of Hematology, December 32, 2011 (12)

[Basic information]

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[medical device registration certificate No. / product technical requirement No.]

Medical device registration certificate No.: gxzz 20193400835

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