Product Cat. No.: FP-314-2

For Clinical Diagnosis & Scientific Research.

Prenatal chromosomes probe detection kit
Instructions Manual

[Product Name] Prenatal chromosomes probe detection kit.
[Package Specifications] 10 Tests/box.

[Intend use]

This kit is mainly used to detect 13/18/21/X/Y chromosome number in amniotic fluid cell samples.
The design of the kit includes two groups of probes, 18/X/Y and 13/21. The probe combination 18/X/y was located in 18p11.1-q11.1, xp11.1-q11.1 and yp11.1-q11.1, respectively, and 13/21 was located in 13q14.2 and 21q22.13 regions, respectively. It is suitable for pregnant women with clinical high risk factors (such as elderly pregnant women, pregnant women with abnormal fetal structure and suspected 13/18/21/X/Y chromosome number abnormality) and the detection results are not used as the basis for clinical diagnosis. This product is only suitable for the detection of amniotic fluid cells, and cannot detect other chromosomal structural abnormalities that can lead to birth defects, and should not be used for gender identification of non-medical needs.

[Detection principle]

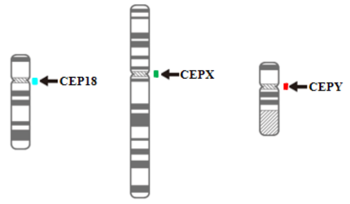
Fluorescence in situ hybridization (FISH) is a technique for directly observing specific nucleic acids in cells in vitro. According to the principle of base complementary pairing, the specific DNA sequence is complementary to the target sequence in the cell. Because the probe has fluorescence, the hybridization probe and target DNA can be clearly observed under the fluorescence microscope under the appropriate excitation light.
The kit consists of two groups of probes, 18/X/Y and 13/21. The probe combination 18/X/y was located in 18p11.1-q11.1, xp11.1-q11.1 and yp11.1-q11.1, respectively, and 13/21 was located in 13q14.2 and 21q22.13 regions, respectively. The probe can be combined with the target site by in situ hybridization, and the corresponding fluorescence signal points in a single cell can be observed clearly under the fluorescence microscope, so as to determine the number of the chromosome segment. The chromosome number of 13/18/21/X/Y in the samples was detected by this method, which can provide reference for clinical diagnosis.

[Product Main Components]

The kit consists of CEP18/CEPX/CEPY Trichromatic probe as shown in Table 1.

Table 1: Kit composition

Component name	Specifications	Quantity	Main components
CEP18/CEPX/CEPY probe	100μL/Tube	1	CEP18 aqua probe, CEPX green probe, CEPY orange red probe



The reagents, instruments and consumables required for the detection of this kit are as follows:
Reagents: absolute ethanol, deionized water, potassium chloride, methanol, acetic acid (glacial acetic acid), 4% paraformaldehyde, magnesium chloride, trypsin, 1m hydrochloric acid, pepsin, 20×PBS（pH7.2-7.4）、20×SSC (pH7.0), NP-40, DAPI counterstaining agent (1.5μg/mL）
Instruments: 15ml centrifuge, 1.5ml centrifuge, pipette (1mL, 100μL, 10μL) , constant temperature water bath, refrigerator (-20ºC±5ºC), baking machine, hybridization instrument, fluorescence microscope (equipped with relevant filter)
Consumables: 15ml centrifuge tube, pipette suction head, anti-drop slide, 22×22mm cover glass, dyeing cylinder, rubber glue, tweezers, timer.

[Storage conditions & Validity]
Keep sealed away from light at -20ºC±5ºC. 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 2-8ºC in dark. For long-term preservation after opening, keep the lid sealed at -20ºC±5ºC away from light. The kit is transported under 0ºC.

[Applicable Instruments]
1. Fluorescence microscopic imaging system, including fluorescence microscope and filter group. The kit is labeled with orange fluorescein and green fluorescein, and the filter group suitable for fluorescent labeling dye shall be selected.
The maximum excitation wavelength is 555nm, and the maximum excitation wavelength is 565nm;
Green fluorescence: the maximum excitation wavelength is 490nm and the maximum emission wavelength is 515nm;
Aqua fluorescence: the maximum excitation wavelength is 423nm and the maximum emission wavelength is 480nm.
The fluorescence microscopic imaging system shall be orange, green and aqua channel microscope; When the microscope is a monochromatic channel, the image synthesis analysis results shall be used.
2. Automatic hybridization instrument: strict temperature uniformity is required, and the temperature difference is ≤ 1℃.
3.

4. [Sample requirements]
1. Fresh amniotic fluid samples shall be stored at 2~8℃ and processed within 7 days;
2. Amniocentesis amniotic fluid cell sample, 16~22 weeks of pregnancy with medical indications + 6;
3. If the amniotic fluid is brown or blood colored, which will affect the accuracy of the results, the sample should be taken again.

[Test method]

1. Related Reagents

1. Related reagents
The following reagents are required for the experiment and are not provided in this kit.
①2×SSC
Take 200ml 20×SSC (pH 7.0), diluted and mixed with 800ml deionized water, stored at 2-8ºC, with a shelf life of 6 months. If the reagent is turbid or contaminated, it cannot be used.
②1×PBS
Take 100ml 20×PBS (pH 7.2-7.4), diluted and mixed with 1900mL deionized water, stored at 2-8ºC, with a shelf life of 6 months. If the reagent is turbid or polluted, it cannot be used.
③ 0.05% trypsin solution
Weigh 0.05g trypsin and use 80ml 1×PBS is dissolved, and the volume is fixed to 100ml after sufficient dissolution -20ºC±5ºC for 12 months.
④ 0.075m KCl solution
Weigh 2.8g potassium chloride and dissolve it with 400ml deionized water. After sufficient dissolution, fix the volume to 500ml. Store at room temperature with a shelf life of 6 months. If the reagent is turbid or contaminated, it cannot be used.

⑤ Stationary solution (methanol: glacial acetic acid = 3:1).
Take 30ml methanol and 10ml glacial acetic acid from the dosage cylinder and fully mix them. Ready to use.
⑥ Pepsin
Pepsin storage solution: weigh 10g pepsin, dissolve it with 80ml deionized water, and then fix the volume to 100ml -20ºC±5ºC for 12 months.
Pepsin working solution: measure 40ml deionized water with a dosage cylinder and suck 400μL 1M hydrochloric acid with a pipette, fully mix and add 100μL pepsin storage solution. Ready to use.
⑦ 1% paraformaldehyde /PBS
Measure 26ml of deionized water and 10ml of 4% paraformaldehyde with a measuring cylinder, and suck 2mL 20×PBS、2mL1M $MgCl_2$ with a pipette, fully mixed. It shall be stored at 2~8℃ for 6 months. If the reagent is turbid or polluted, it cannot be used.
⑧ Ethanol solution: 70% ethanol, 90% ethanol
Dilute 700ml and 900ml of absolute ethanol to 1L with deionized water respectively. The shelf life is 6 months. If the reagent is turbid or polluted, it cannot be used.
⑨0.3%NP-40/0.4×SSC solution, pH 7.0 ~ 7.5
Measure 150ml deionized water with a measuring cylinder and suck 4mL 20×SSC 0.6mLNP-40 with a pipette, fully mix, adjust the pH value to 7.0~7.5 at room temperature, fix the volume to 200ml with deionized water, store at 2~8℃, and the shelf life is 6 months. If the reagent is turbid or polluted, it cannot be used.
⑩ For diamidino phenylindole (DAPI) counterstaining agent, please choose commercial DAPI counterstaining agent containing anti quenching agent.

2. Sample collection and slides preparation
① Aspirate ≥5ml sample into 15ml centrifuge tube, centrifugation, 2000 rpm, 10min, remove the supernatant (the sample cannot show blood color or brown), and leave about 1ml at the bottom of the tube;
② 5 ml of 1xPBS was added into the precipitate and shaken, centrifuged at 2000 rpm for 10 min at room temperature. The supernatant was discarded and about 1ml was left at the bottom of the tube;
③ Add 5ml 0.05% trypsin solution for shaking and mixing, place at 37ºC for 10 min, centrifuge at 2000 rpm for 10 min at room temperature, discard the supernatant, and leave about 1ml at the bottom of the tube;
④ Add 5ml 0.075mol/l KCl (preheated at 37ºC before use), gently mix it upside down, and incubate at 37ºC for 20min.
⑤ Slowly stick to the wall and add 1ml of stationary solution (prepared by waiting time in the previous step, methanol: glacial acetic acid = 3:1 volume ratio), let stand at room temperature for 10min, centrifuge at 2000rpm for 10min, and discard the supernatant to about 1ml;
⑥ Add 5ml fixed solution to the precipitation, shake and mix well, and put it into the refrigerator at -20±5℃ for at least 1 hour;
⑦ Centrifuge at 2000 rpm for 10 minutes at room temperature and suck up the supernatant as much as possible.

3. Slide preparation
Add the fixed solution according to the amount of cell precipitation, blow and mix well, suck 2ul cell suspension and drop it onto the anti-detachment slide, and adjust the cell concentration through microscopic examination (2ul drop each time to enrich the cells until the cell amount is enough). The number of cells in a single field of view of the sample under 10 times microscope should be 10~50, and each sample should be dropped with two slides.

4. Slide pretreatment procedure:
① After baking at 56ºC for 30 min,
②At room temperature, place the slide at 37℃ 1×PBS for 5 minutes;
③protease working solution (prepared when using) preheated at 37ºC was used for digestion, and the digestion time was 10 min (the time should be extended or shortened according to the sample situation).
④ Washing with 1xPBS at room temperature for 3min.
⑤ Take out the glass slide and fix it in 1% paraformaldehyde/PBS for 10 minutes at room temperature.
⑥ Wash with 1×PBS for 3min.

⑦ They were placed in 70%, 85% and 100% ethanol for 2 min respectively.
⑧ The slides were taken out and dried at room temperature.

5. Denaturing hybridization

The following operations should be carried out in a dark room.
①Take out the 13q14.2/21q22.13 and CEP18/CEPX/CEPY probes, let them stand at room temperature for 5 minutes, flick the bottom of the centrifuge tube with fingers, mix the probes, and centrifuge briefly. Take 10μL drops into the hybridization area of the cell drop slides, and cover them with a 22mmx22mm cover glass immediately. The probes should be evenly spread under the cover glass without bubbles, and the edges should be sealed with rubber glue (the edge sealing must be thorough to prevent dry slides from affecting the test results during hybridization).
②Place the glass slides in the hybridization instrument, denature at 88°C for 2 minutes (the hybridizer should be preheated to 88°C) and hybridized at 45°C for 2-16 hours.

6. Washing

The following operations should be carried out in a dark room.
① Use tweezers to carefully tear off the sealing glue around the cover glass to avoid sticking or moving the cover glass. Immerse the glass slide in 2xSSC for about 5s and take it out. Gently push one corner of the cover glass to the edge of the slide with tweezers, and gently remove the cover glass with tweezers;
②The slides were placed at 2xSSC room temperature for 1 min;
③The slides were immersed in 0.3% NP-40/0.4×SSC solution preheated at 68°C for 2 min;
④Take out the slide and immerse it in deionized water preheated at 37°C in advance for 1 min, and then dry naturally in the dark.

7. Counterstaining

The following operations should be carried out in a dark room.
Drop 10μL DAPI dye on the hybridization area, cover the slide immediately, and select the appropriate filter to observe the slide under the fluorescence microscope.

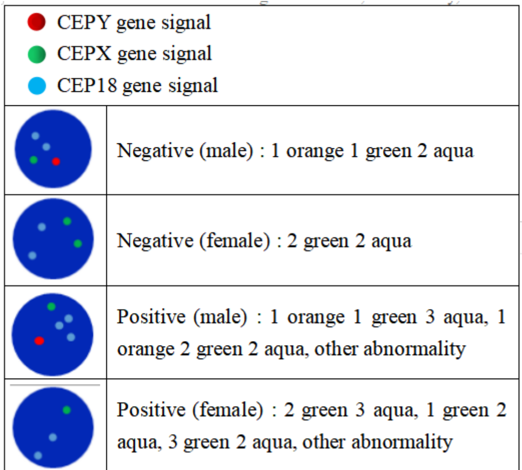
8. FISH results observation

① Results observation method: put the counterstained glass slide under the fluorescence microscope and put it under the low power objective lens (10x) confirm the cell area under the microscope; Go to 40x under the objective lens, find a position where the cells are evenly distributed; Then in the high-power objective (100x) the FISH results of nuclei were observed.
② Interpretable sample standard: the hybridization signal of the probe is bright and clear, the orange, green and aqua signals are easy to distinguish, the spontaneous fluorescence does not affect the signal count, and the number of countable cells is not less than 50.
③ Standard of countable cells: the cells are reasonably distributed without overlap, DAPI counterstaining is clear, that is, the nuclear boundary is clear, and there is at least one signal (orange, green and aqua signal) in each cell.
④ Counting method: randomly count 50 cells in each sample and count the number of orange, green and aqua signals in each nucleus.

[Positive judgment value or reference interval]

1. Common signal classification

Table 2: Classification of CEP18/CEPX/CEPY common signal and judgment of cell negative and positive



CEPY gene signal CEPX gene signal CEP18 gene signal	
	Negative (male) : 1 orange 1 green 2 aqua
	Negative (female) : 2 green 2 aqua
	Positive (male) : 1 orange 1 green 3 aqua, 1 orange 2 green 2 aqua, other abnormality
	Positive (female) : 2 green 3 aqua, 1 green 2 aqua, 3 green 2 aqua, other abnormality

2. Threshold setting

Referring to the result judgment criteria in the "expert consensus on the application of fluorescence in situ hybridization in prenatal diagnosis" and Abbot's "aneu vysionmulticolor DNA Probe Kit", when the proportion of normal cells is ≥90% and the proportion of abnormal cells is <10%, it indicates that the index is normal (negative); When the proportion of abnormal cells in a certain index is≥10%, it indicates that the index is abnormal (when the proportion of abnormal cells is between 10%~60%, it indicates chimerism, and when the proportion of abnormal cells is ≥60%, it is judged as positive).

[Interpretation of test results]

1. Result judgment

CEP18/CEPX/CEPY three color probe: count and analyze 50 nuclei continuously in each sample, and record the number of chromosome signal points (1,2,3,4, > 4). The report form is to record the percentage of abnormal signal cells in 50 cells of three color signals respectively; In case of chimerism (10 ~ 60% abnormal cells), the sample needs to count and analyze 100 nuclei, and then give the percentage of abnormal cells.

2. Judgment of invalid or unreliable results of the experiment:

① If the number of cells available for analysis is less than 50, the test samples shall be supplemented or 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 the result, this detection shall be judged as unreliable and treated as invalid;

③ If the test sample is considered to be chimera (the proportion of abnormal cells is between 10% and 60%), 100 nuclei shall be counted.

3. The common factors affecting the test results and the treatment methods are shown in Table 3

Question	Possible Cause	Recommended Solution
Too strong background	Slides were not cleaned properly before specimen's preparation.	Slides washing with anhydrous ethanol.
	Incomplete washing after hybridization.	Ensure that the washing solution is prepared according to instructions; make sure that the washing solution pH and temperature are correct; remove the coverslip and repeat the washing.
	Filter sets improper use	Replace the appropriate filter set to weaken the background light.
	Improper hybridization conditions.	Ensure that the hybridization instrument temperature is 42ºC
	Low washing temperature.	Ensure that the solution temperature of the washing glass slides is up to the required temperature.
	Washing solution strength is too low.	Ensure that the washing solution is prepared according to instructions. (Low SSC concentration and high NP-40 concentration are beneficial to increase the washing solution strength).
The dye is too weak	Distaining	Remove coverslips and soak for 5 minutes in a 2xSSC/0.1% NP-40 washing solution at room temperature. Place the slides sequentially in 70%, 85%, and 100% ethanol solutions for 1 minute each for gradient dehydration and then re-dye.
	Obsolete dye agent or Excessive illumination	Ensure that the dye agent is stored at -20ºC to avoid light, and ensure that the dye agent is not invalid.
No signal or weak signal	Sample incomplete denaturation	Ensure that the hybridization instrument temperature is 83ºC, preheat it for at least 10 minutes in advance.
	Incomplete mix before use of the probe and hybridization buffer	Blow the probe mixture and mix the probe thoroughly. Centrifuge briefly.
	The probe mixture dries too fast on the glass slide	The target area should be immediately covered after the probe mixture is dropped with cover glass; when washing, only one cover glass on the slide can be removed at a time and the slide can be immersed in the washing solution immediately before the next one is removed.
	Bubble formation under cover glass during hybridization.	Cover the surface of the probe mixture and gently squeeze to release the bubbles.
	Inappropriate hybridization conditions	Ensure that specified hybridization time and temperature are observed; that no gaps are left in the rubber seal, and that the time of hybridization is adjusted.
	Incorrect washing or inappropriate washing conditions	Ensure that the washing solution is prepared according to the product specification; ensure that the temperature of the washing solution reaches the specified temperature for the washing step; ensure that the thermometer and pH meter are correctly calibrated; remove the cover glass before the slide is immersed in the washing solution.
	Inappropriate probe storage or specimen slides	Ensure that probes are stored in the dark at -20°C. Place the unhybridized slides dry at -20°C for a long storage period of time or at room temperature for a short storage period of time. After hybridization, store in dark the (hybridized) slides at -20°C. The storage period should not exceed 6 months.
	Dye agent incorrect usage Dye agent too high brightness	Remove the coverslip and soak the slides in 2xSSC/0.1% NP-40 solution for 5 minutes at room temperature. Place slides sequentially in 70%, 85% and 100% ethanol solutions for 1 minute each to dehydrate. Dry the slides naturally and add the dye agent
	Inappropriate filter set selection for observation	Use the appropriate filter set to observe the fluorescence of the probe.

[Limitations of test methods]

This kit is used for fresh amniotic fluid samples, and is not recommended for other types of cells or tissues. It should be operated according to the procedures provided in this manual. Changing the procedures may change the test results.

This kit is only suitable for the detection of 13/18/21/X/Y chromosome number abnormality, and cannot detect other chromosomal abnormalities. The detection results are only for clinical reference, not as the basis for clinical diagnosis alone. Clinicians should make a comprehensive judgment on the test results in combination with other detection indicators of pregnant women.

[Product performance index]

1. Appearance: the outer package of the kit shall be complete without damage, and the marks shall be complete and clear; All liquid reagents shall be clearly marked without leakage.
2. Fluorescence signal intensity: after the probe is effectively hybridized with the karyotype reference, it should send out a fluorescence signal that can be recognized by the naked eye under the fluorescence microscope.
3. Sensitivity: after effectively hybridizing with karyotype reference, CEP18/CEPX/CEPY trichromatic probe analyzed 100 chromosomes 18 of 50 cells in metaphase division phase, and at least 98 chromosomes 18 showed 1 cyan fluorescence signal; 100 chromosomes X of 100 cells in metaphase division phase were analyzed, and at least 98 chromosomes X showed a green fluorescence signal; 100 chromosomes Y of 100 cells in metaphase were analyzed, and at least 98 chromosomes Y showed an orange fluorescence signal.

13q14. 2/21q22. After effectively hybridizing with the karyotype reference, 100 chromosomes 13 of 50 cells in metaphase were analyzed, and at least 98 chromosomes 13 showed 1 green fluorescence signal; 100 chromosomes 21 of 100 cells in metaphase were analyzed, and at least 98 chromosomes 21 showed an orange fluorescence signal.

4. Specificity: after effectively hybridizing with karyotype reference, CEP18/CEPX/CEPY three-color probe analyzed 100 chromosomes 18 of 50 cells in metaphase division phase, and at least 98 chromosomes 18 showed a specific cyan fluorescence signal in the target region of centromere; 100 chromosomes X of 100 cells in metaphase division phase were analyzed, and at least 98 chromosomes X showed a specific green fluorescence signal in the centromeric target region; 100 chromosomes Y of 100 cells in metaphase division phase were analyzed, and at least 98 chromosomes Y showed a specific orange fluorescence signal in the centromeric target region.

13q14. 2/21q22. After effectively hybridizing with the karyotype reference, the two-color probe analyzed 100 chromosomes 13 of 50 cells in metaphase division phase, and at least 98 chromosomes 13 showed a specific green fluorescence signal in the long arm target area; 100 chromosomes 21 of 50 cells in metaphase were analyzed, and at least 98 chromosomes 21 showed a specific orange fluorescence signal in the target region of the long arm.

5. Coincidence rate of negative and positive: detect 5 negative reference materials and analyze the fluorescent signal. The results should meet the negative judgment standard and are all judged to be negative. Five positive reference samples were tested and the fluorescence signals were analyzed. The results should meet the positive judgment criteria and were all judged to be positive.

[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.
2. When testing clinical samples, when the hybridization signal counting is difficult and the sample is not enough to repeat the retest, or the cell volume is not enough for analysis, the test will not provide the test results.
3. DAPI counterstaining agent used in this experiment has potential toxicity or carcinogenicity, so it is necessary to operate in the fume hood, wear masks and gloves to avoid direct contact.
4. All chemicals are potentially dangerous. Avoid direct contact. Used kits are clinical waste and should be properly disposed off.

[References]

[1]. Prenatal screening and diagnostic criteria for common fetal chromosomal abnormalities and open neural tube defects Part 2: cytogenetic prenatal diagnostic criteria for fetal chromosomal abnormalities. Zheng Y L , Ferguson-Smith M A , Warner J P , et al. Analysis of chromosome 21 copy number in uncultured amniocytes by fluorescence in situ hybridization using a cosmid contig[J]. Prenatal Diagnosis, 1992, 12(11):931-943.

[2]. Ried T , Landes G , Dackowski W , et al. Multicolor fluorescence in situ hybridization for the simultaneous detection of probe sets for chromosomes 13, 18, 21, X and Y in uncultured amniotic fluid cells.[J]. Human Molecular Genetics, 1992, 1(5):307.

[3]. Ward B E , Gersen S L , Carelli M P , et al. Rapid Prenatal Diagnosis of Chromosomal Aneuploidies by Fluorescence in Situ Hybridization: Clinical Experience With 4500 Specimens[J]. Obstetrical and Gynecological Survey, 1994, 49(3):163-165.

[4]. Cacheux V, Tachdjian G, Druart L, et al. Evaluation of X, Y, 18, and13/21 alpha satellite DNA probes for interphase cytogenetic analysis of uncultured amniocytes by fluorescence in situ hybridization. *PrenatDiagn.* 1994;14:79-86.

[Basic information]

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[Manual Approval date & Revision date]

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