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Product Catalogue Number FP-314 **For clinical diagnosis and scientific research.**

Prenatal Chromosomes Probe Detection Kit

[Product Name] Prenatal chromosomes probe detection kit (Fluorescence In Situ Hybridization Method).

[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.

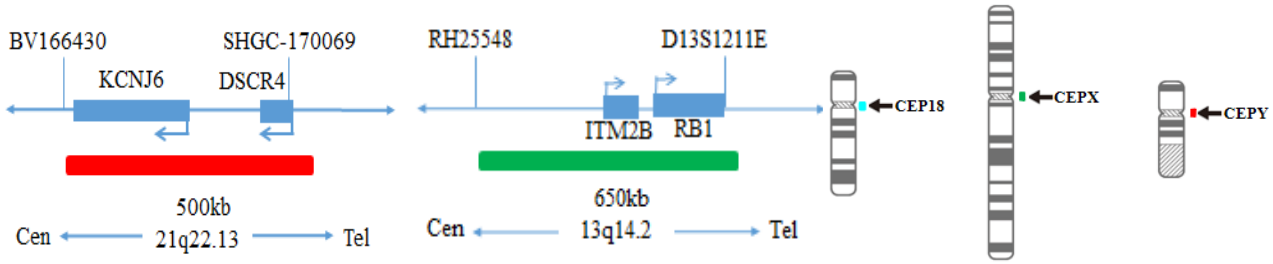
[Detection principle]
Fluorescence in situ hybridization (FISH) is a technique for directly observing specific nucleic acids in cells in vitro. According to the principle of base complementary pairing, the specific DNA sequence is complementary to the target sequence in the cell. Because the probe has 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 18/X/Y Trichromatic probe and 13/21 dual color probe as shown in Table 1.

Table 1 Kit composition

Component name	Specifications	Quantity	Main components
18/X/Y trichromatic probe	100μL/Tube	1	18 Cyan probe, X Green probe, Y Orange red probe
13/21 dual color probe	100μL/Tube	1	13 Green probe, 21 Orange red probe



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

[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 hybridizer: strict temperature uniformity is required, temperature difference ≤1°C.

[Sample requirements]

1. Amniocentesis amniotic fluid cell samples, pregnant 16-23 weeks with medical indications;
2. Fresh amniotic fluid samples were stored at 2 ~ 8℃ for 7 days;
3. If the amniotic fluid is brown or bloody (mother source pollution), the accuracy of the results will be affected, so the sample should be taken again.

[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 2-8oC, the solution shelf life is of 6 months. Discard if the reagent appears cloudy (turbid) or contaminated.

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°C, 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

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°C, the shelf life is of 6 months. Discard if the reagent appears cloudy (turbid) or contaminated.

⑤Protease working solution: 5mg pepsin was added into 50ml 0.01N HCl solution, and then prepared and used.

⑥ 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.

⑦PBS buffer, pH 7.4 ± 0.2

Weigh 8g Sodium chloride, 0.2g Potassium chloride, 3.58g Sodium hydrogen phosphate , 0.27g Potassium dihydrogen phosphate. Dissolve the above reagents with 800ml deionized water, adjust the pH value to 7.4±0.2 at room temperature, and fix the volume to 1L with deionized water. It can be stored at room temperature for 6 months. If the reagent is turbid or contaminated, it cannot be used.

⑧ 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.

⑨ Configuration of post fixed solution: measure 48.7ml 1xPBS, add 481 μl formaldehyde solution, add 0.46g MgCl2·6H2O, mix well, and prepare for current use.

⑩ Diamidinyl phenylindole (DAPI) counterstain

Use commercially available anti-quenching DAPI counterstain.

2. Sample collection and slides preparation

① Sample collection: fresh amniotic fluid samples.

② Aspirate ≥5ml sample into 15ml centrifuge tube, centrifugation, 2000rpm, 10min, remove the supernatant (the sample cannot show blood color or brown), and leave about 1ml at the bottom of the tube.

③ 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 1 ml was left at the bottom of the tube.

④ Add 5ml 0.05% trypsin solution (1xPBS, preheated at 37°C before use) for shaking and mixing, place at 37°C 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.
⑤ Add 5ml 0.075mol/l KCl (preheated at 37°C before use), gently mix it upside down, and incubate at 37°C for 20min.
⑥ 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 5min, centrifuge at 2000rpm for 10min, and discard the supernatant to about 1ml.
⑦ Add 5ml of stationary solution into the precipitate, shake and mix well, put it into the refrigerator at -20°C for at least 1 h, centrifuge at room temperature at 2000 rpm for 10 min, and absorb the supernatant as much as possible.
⑧ Before dropping, the concentration of cell suspension was adjusted according to the amount of precipitation;
⑨ The cells suspension was directly dropped onto 1-2 slides to form two hybridization regions.

3. Slide pretreatment procedure:
① After baking at 56°C for 30 min, the samples were placed at 2xSSC (37°C) for 5 min;
② Fresh protease working solution preheated at 37°C was used for digestion, and the digestion time was 10 min (the time should be extended or shortened according to the sample situation).
③ Washing with 1xPBS at room temperature for 5 min.
④ Post fixation: after being placed in the fixed solution, it was treated at room temperature for 5 min.
⑤ Wash with 1xPBS for 5min and air dry.
⑥ They were placed in 70%, 85% and 100% ethanol for 1 min respectively.
⑦ The slides were taken out and dried at room temperature.

4. Denaturing hybridization
① The following operations should be carried out in a dark room.
Take out the 13/21 and 18/X/Y 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).
② The slides were placed on the hybridizer and co denatured at 88°C for 5 minutes (the hybridizer should be preheated to 88°C) and hybridized at 45°C for 2-16 hours.

5. 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 5 s 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.
② The slides were placed at 2xSSC room temperature for 1 min;
③ The slides were immersed in 0.3% NP-40/0.4×SSC solution preheated at 68°C for 2 min.
④ Take 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.

6. Complex dyeing
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.

7. FISH results observation
The stained slides were placed under the fluorescence microscope, and the cell regions were identified under the low power objective (10x), and then the fish results of the nucleus were continuously observed from the upper left to the lower right under the high power objective (100x). When there are fluorescence signal points outside the cell, it is better to distinguish it from the intracellular fluorescence signal points. It is better to count 50 cells in each sample randomly. The signal number of related chromosomes in each nucleus is counted, and the percentage of abnormal cells with abnormal chromosome number is calculated. If the effective number of cells in the sample is less than 50, a sample piece should be added to continue counting or the sample information is insufficient; if chimerism occurs, another 50 cells need to be counted.

[Positive judgment value or reference interval]

1. Common signal classification

Table 2: 13/21 common signal classification and cell negative positive judgment

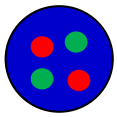
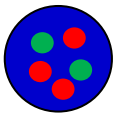
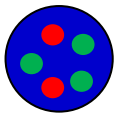
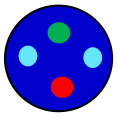
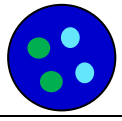
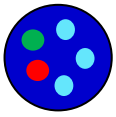
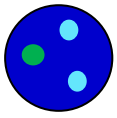
21q22.13 signal 13q14.2 signal	
	**Negative**: 2 Orange ; 2 Green
	**Positive**: 3 Orange ; 2 Green, other anomaly
	**Positive**: 2 Orange ; 3 Green, other anomaly

Table 3: Classification of CEP18 / CEPX / CEPY common signal and judgment of cell negative and positive

CEPY signal CEPX signal CEP18 signal	
	**Negative** (Male): 1 Orange ; 1 Green ; 2 Cyan
	**Negative** (Female): 2 Green ; 2 Cyan
	**Positive (Male):** 1 Orange ; 1 Green ; 3Cyan 1 Orange ; 2 Green ; 2 Cyan 1 Orange ; 2 Cyan ; Other anomaly
	**Positive(Female)**: 2 Green ; 3 Cyan ; 1 Green 2 cyan 3 Green ; 2 Cyan ; Other anomaly

2. Threshold setting

According to Abbot "Aneu VysionMulticolor DNA Probe Kit" According to the reference value standard in the instruction manual of kit, and verified by clinical samples, the threshold value of this kit is set as follows: if the proportion of aneuploid cells is greater than 60%, it is judged as aneuploidy; if the proportion of aneuploid cells is less than 10%, it is determined as aneuploid; if the proportion of aneuploid cells is between 10% and 60%, it is considered to be chimerism, which should be comprehensively judged in combination with other relevant detection.

[interpretation of test results]

1. Count 50 cells, count the number of positive and negative cells according to the positive and negative cell judgment method shown in Table 2 and table 3, count each cell once, only count the cells with hybridization signal (at least one color signal), do not count if there is no signal, and do not count the cells with weak signal or too diffuse signal.

① If two signals are close in distance and similar in size but not connected together, they are counted as two signals;
② If the signal is diffuse, but the diffuse part is concentrated and the boundary is clear, count one signal;
③ If two signals are connected by a visible connection, one signal is counted;
④ The nuclei with 0, 1, 2, 3, 4 or more than 4 signals were counted, and only the nuclei with at least one signal were counted. If there is any doubt about the accuracy of counting, the counter is counted again in another area of the slice;
⑤ If the signal is uncertain, it is not counted.

2. Judgment of invalid or unreliable results of the experiment

① If the number of cells available for probe analysis is less than 50, the sample should be supplemented or the test should be invalid;
② If the intensity or background of fluorescence hybridization signal available for analysis is not ideal or clear, which affects the judgment of results, this detection should be judged as unreliable and treated as invalid experiment;
③ If the test sample is considered to be chimerism (the proportion of aneuploid cells is between 10% and 60%), 100 nuclei should be counted.

3. The common factors affecting the test results and the treatment methods are shown in Table 4

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.
Question	**Possible Cause**	**Recommended Solution**
Too strong background	Improper hybridization conditions.	Ensure that the hybridization instrument temperature is 42°C
	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).
Question	**Possible Cause**	**Recommended Solution**
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 -20°C to avoid light, and ensure that the dye agent is not invalid.
Question	**Possible Cause**	**Recommended Solution**
No signal or weak signal	Sample incomplete denaturation	Ensure that the hybridization instrument temperature is 83°C, 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.
Question	**Possible Cause**	**Recommended Solution**
No signal or weak signal	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 can not 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.

[Precautions]

1. Please read this manual carefully before testing. The testing personnel shall receive professional technical training. The signal counting personnel must be able to observe and distinguish orange red and green signals.
2. When testing clinical samples, if it is difficult to count the hybridization signals 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, again, the test will not provide test results.
3. The formamide and DAPI counterstaining agent used in this experiment have potential toxicity or carcinogenicity, so they need to be operated 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 enzyme digestion time, hybridization temperature and time, operating environment and limitations of current molecular biology technology, which may lead to wrong results. The user 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 wastes and should be properly disposed of.
6. This product is for clinical diagnosis and scientific research.

[Manuscript version and approval date]

Manual version: V1.2
Approval date: 21 December 2021