

Prenatal Chromosomes Probe Detection Kit (CW-314)

Intended 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.

Product composition

The kit consists of 18/X/Y Trichromatic probe and 13/21 dual color probe as shown below.

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

Storage condition

Keep sealed away from light at $-20^{\circ}\text{C}\pm 5^{\circ}\text{C}$. 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 $2-8^{\circ}\text{C}$ in dark. For long-term preservation after opening, keep the lid sealed at $-20^{\circ}\text{C}\pm 5^{\circ}\text{C}$ away from light.

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;

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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 $\leq 1^{\circ}\text{C}$.

Sample requirements

1. Amniocentesis amniotic fluid cell samples, pregnant 16-23 weeks with medical indications.
2. Fresh amniotic fluid samples are stored at $2 \sim 8^{\circ}\text{C}$ 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.

Related reagents

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 $2-8^{\circ}\text{C}$, 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 $2-8^{\circ}\text{C}$, 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

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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.

5. Protease 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.

7. PBS 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.

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.

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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.

Slide pretreatment procedure

1. After baking at 56°C for 30 min, the samples were placed at 2xSSC (37°C) for 5 min.
1. 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).
2. Washing with 1xPBS at room temperature for 5 min.
3. Post fixation: after being placed in the fixed solution, it was treated at room temperature for 5 min.
4. Wash with 1xPBS for 5min and air dry.
5. They were placed in 70%, 85% and 100% ethanol for 1 min respectively.
6. The slides were taken out and dried at room temperature.

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

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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.

Washing

The following operations should be performed in a darkroom.

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-40/0.4xSSC 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.

Counterstaining

The following operations should be performed in a darkroom.

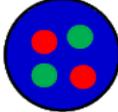
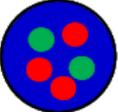
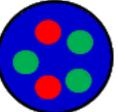
10µL DAPI compound dye is dropped in the hybridization area of the glass slide and immediately covered. The suitable filter is selected for glass slide observation under the fluorescence microscope.

FISH results observation

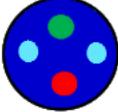
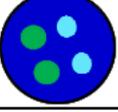
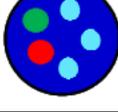
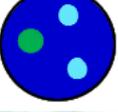
The stained slides were placed under the fluorescence microscope, and the cell regions were identified under the low power objective (10x), and then the fish results of the nucleus were continuously observed from the upper left to the lower right under the high power objective (100x). When there are 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.

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13/21 common signal classification and cell negative positive judgement

	● 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	

Classification of CEP18/CEPX/CEPY common signal and judgement 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		

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 above tables, 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.
2. If two signals are close in distance and similar in size but not connected together, they are counted as two signals.
3. If the signal is diffuse, but the diffuse part is concentrated and the boundary is clear, count one signal.
4. If two signals are connected by a visible connection, one signal is counted.
5. 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.
6. If the signal is uncertain, it is not counted.

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.

Frequent problems and solutions

Problem	Possible cause	Recommended solution
Strong background of slides	Inadequate wash of glass slide before preparation of specimens	Wash the glass slide using the absolute ethyl alcohol
	Inadequate wash after hybridization	Assure that the wash buffer is prepared in line with Instruction For Use, assure the correct pH value and temperature of wash buffer, remove the coverslip and repeat the washing steps
	Improper use of filter sets	Replace with suitable filter sets to reduce the background light
	Improper hybridization condition	Assure the temperature of hybridization instrument is set as 42°C

	The temperature is too low when washing	Assure that the wash buffer reaches to the required temperature when washing the slides
	The washing intensity of wash buffer is too low	Assure the wash buffer is prepared in line with Instruction For Use. (low SSC concentration or high NP-40 concentration would help improving the washing intensity of wash buffer)
Weak counterstaining	Weak counterstaining	Remove coverslip, at room temperature, immerse the slides in the wash buffer containing 2 × SSC/ 0.1%NP-40 for 5 minutes. And then sequentially immerse the slides in 70%, 85% and 100% ethanol solution for 1 minutes respectively, and then perform the counterstaining
	The counter stain has been kept under long-term storage or excessive light	Assure the counter stain is stored at -20°C and protected away from light, assure its effect
No signal or weak signals	Inadequate denaturation of specimens	Assure the temperature of hybridization instrument is set as 83°C, at least 10 minutes in advance is needed to preheat hybridization instrument
	The probe mixture and hybridization buffer were not mixed sufficiently before use	Blow the probe mixture and mix the probe sufficiently, centrifuge for a short time
	The probe mixture on tissue slides dries too fast	After dropping probe mixture the target area should be covered by coverslip immediately, when washing the slides you can only remove one coverslip at a time, and dip it into wash buffer immediately before removing next coverslip
	Air bubbles formed under coverslip during hybridization	The coverslip should cover the probe mixture in order to gently squeeze out air bubbles
	Inappropriate hybridization condition	Ensure to comply with the time and temperature required by hybridization and do not leave gaps when sealing the slides with rubber cement. The hybridization time should be adjusted according to the situation.
	Improper wash buffer or incorrect washing conditions	Be sure to follow the requirements of Instruction for Use to formulate the wash buffer. Ensure that the temperature of wash buffer reaches to the temperature predetermined in washing step. The thermometer and pH meter should be accurately calibrated. Remove coverslip before immersing the slide into wash buffer
	Inappropriate storage of probe or specimens slides	Make sure that the probe mixture is stored at -20°C and protected from light. Place the slides without hybridization at -20°C for long-term storage or at room temperature for short-term storage. Place the hybridized slides at -20°C, away from light, and store for less than 6 months
	Incorrect use of DAPI counter stain, excessively high brightness of counter stain	Remove the coverslip, immerse the slides in 2 × SSC/ 0.1%NP-40 for 5 minutes at room temperature.

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		Sequentially immerse the slides in 70%, 85% and 100% ethanol solution for 1 minutes respectively, and then perform the counterstaining after air drying the slides
	Inappropriate filter sets were selected for observation	Use correct filter sets to observe the probe fluorescence

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]

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