Chapter 4

In Situ Hybridization of Breast Cancer Markers

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Abstract

In situ hybridization is an important technique in breast cancer research, which is widely applied in detection of specific nucleic acid sequences. Here, we describe the detailed protocol of fluorescence in situ hybridization and chromogenic in situ hybridization in detection of gene HER2/*neu* amplification in breast cancer tissues.

Key words In situ hybridization, Breast cancer, HER2, FISH, CISH

1 Introduction

Recently, in situ hybridization is widely applied in detection of specific nucleic acid sequences [1–3].

The mechanism of in situ hybridization is based on complementarity of single-strand nucleic acid [1]. The procedure of in situ hybridization can be summarized as three main steps: preparing radioactive or nonradioactive targeted exogenous nucleic acid (i.e. probe), hybridizing pretreated sample DNA or RNA on the tissue, cell, or chromosome with probes, detecting and mapping the specific hybrid nucleic acid [4, 5].

In breast cancer research, many types of hybridization methods have been used, such as Dot blot, Southern blot, Northern blot, Tissue in situ hybridization, Genome in situ hybridization [2, 6]. Based on different probes and visualization reagents, in situ hybridization can be classified into fluorescence in situ hybridization (FISH), chromogenic in situ hybridization (CISH), radiolabeled in situ hybridization, dual-color silver-enhanced in situ hybridization and et al [7–10]. FISH and CISH are the methods being most widely used in breast cancer research.

Human epidermal growth factor receptor 2 (HER2) is one of the most three important breast cancer markers [11, 12]. HER2 gene amplification occurs in more than 20 % breast cancer cases

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and was identified as an important prognostic biomarker [13]. HER2 gene amplification can be identified by many different methods, and FISH has become the "Gold Standard" of HER2 gene amplification detection in clinical practice [14, 15]. Recently, CISH was gradually widely studied and used as a viable alternative to FISH in HER2 gene amplification detection [16–18]. In this chapter, we summarized regular protocol of FISH and CISH in detection of HER2, as representative protocols of in situ hybridization of breast cancer markers.

2 Materials

Use ultrapure water (purified deionized water, sensitivity: 18 MΩ·cm at 25 °C) to prepare all buffers and other solutions. Store all solutions at room temperature (about 25 °C) unless otherwise indicated. All reagents used in the experiment should be analytical grade pure unless otherwise indicated.

2.1 Equipment and Reagent

1. 100-W Epifluorescence microscope with appropriate filters (*see* **Note 1**).
2. Water baths set.
3. Microwave oven with appropriate size (for heat pretreatment).
4. Thermal plate set.
5. Silanized microscope slides.
6. "Probe check" quality control slides (Abbott Inc., UK), (*see* **Note 2**).
7. Reveal target unmasking solution (Biocare Inc., Walnut Creek, CA).
8. Staining dishes with 100 % xylene.
9. Staining dishes with 100 %, 90 %, 80 %, 70 % ethanol (*see* **Note 3**).
10. Phosphate buffered saline (PBS), pH 7.4: 0.01 M phosphate buffer, 0.0027 M KCl, 0.137 M NaCl. Dissolve 8 g NaCl, 0.2 g KCl, 1.42 g $Na_2HPO_4{\cdot}2H_2O$, 0.27 g KH_2PO_4 in 800 mL ultrapure water, adjust pH to 7.4 with 10 M NaOH, make up to 1 L with ultrapure water and then autoclave.
11. 2× SSC solution, pH 7.0: 3 M NaCl, 0.3 M sodium citrate. Dissolve 175.3 g NaCl and 88.2 g sodium citrate ($C_6H_5Na_3O_7{\cdot}2H_2O$) in 800 mL ultrapure water, adjust pH to 7.0 with 10 M NaOH, make up to 1 L with ultrapure water and then autoclave, dilute 1:10 with ultrapure water for 2× SSC.

12. Vectashield DAPI solution (Vectorlabs, UK), with 200 ng/mL of 4,6-diamidino-2 phenylindole-2 hydrochloride (Sigma, UK) added.
13. Omnislide hybridization platform (Thermo-Hybaid) with dark plastic lid.
14. 8 % (w/v) Sodium thiocyanate (*see* **Note 4**).
15. Pepsin solution (Digest-All III; Zymed Inc.), (*see* **Note 5**).

2.2 Fluorescence In Situ Hybridization Components

1. HER2/chromosome 17 probe mixture (Pathvysion™ kit).
2. Denaturing solution, pH 7.0: Mix 49 mL Ultrapure formamide (Fluka, UK), 7 mL 2× SSC, with 14 mL distilled water, and then adjust pH to 7.0 with 10 M NaOH.
3. 0.5× SSC: Autoclave 2× SSC solution, dilute 1:4 with ultrapure water for 0.5× SSC.
4. Temporary "coverslips": Cut Parafilm to make temporary coverslips. Temporary "coverslips" are made by cutting regular parafilm into appropriate size, and then add the denaturation solution.
5. 22 × 22 mm slip.
6. Rubber cement.

2.3 Chromogenic In Situ Hybridization Components

1. A ready-to-use probe mixture for HER2 (SpotLight probe series; Zymed Inc., CA).
2. CISH-UnderCover Slips (Zymed Inc.).
3. 0.5× SSC: Autoclave2× SSC solution, dilute 1:4 with ultrapure water for 0.5× SSC.
4. Hematoxylin solution: There are various methods of hematoxylin dye preparation. The method of Heidenhain [19] is preferred for CISH.
5. Mouse anti-digoxigenin antibody (Roche Biochemicals).
6. Powervision + detection kit (ImmunoVision Inc., CA).

3 Methods

All procedures should be performed at room temperature unless expressly indicated.

3.1 Basic Sample Handling

1. Cut tissue to 5-μm sections on silanized slides and then bake at 60 °C overnight. Store sections at room temperature (about 25 °C) until required (*see* **Note 6**).
2. Immerse slides in xylene for 10 min (*see* **Note 7**), twice.
3. Transfer slides into 100 % ethanol for 5 min, twice.

4. Rehydrate slides in graded ethanol (90 %, 80 %, 70 % ethanol, each for 1 min).
5. Place slides in 0.2 N HCl for 20 min (*see* **Note 8**).
6. Wash slides in distilled water for 5 min.
7. Wash slides in PBS wash buffer for 5 min, twice (*see* **Note 9**).
8. Place slides in 8 % sodium thiocyanate (Vysis, UK or Sigma, UK) in distilled water at 80 °C for 30 min (*see* **Note 4**).
9. Wash slides in distilled water for 1 min.
10. Wash slides in PBS wash buffer for 5 min, twice (*see* **Note 9**).
11. Place in protease buffer at 37 °C for 20 min (*see* **Note 10**).
12. Immerse slides in 2× SSC buffer for 5 min, twice.
13. Dehydrate slides in graded ethanol (70 %, 80 %, 90 %, 100 % ethanol, each for 1 min), (*see* **Note 3**).
14. Air-dry slides in an oven at 50 °C.
15. Apply 10 μL DAPI in mountant and cover the slides by temporary coverslips.
16. Assess digestion of tissue with a filter specific for DAPI (*see* **Note 11**).
17. Place all slides in 2× SSC buffer until the temporary coverslips fall off.
18. Air-dry slides in an oven at 50 °C.

3.2 Fluorescence In Situ Hybridization

All procedures in this section should be performed after completed Basic Sample Handling procedures.

1. Apply 100 μL denaturing solution to each slide in a fume hood. Cover with temporary coverslips.
2. Denature slides for 5 min at 94 °C.
3. Remove temporary coverslips in a fume hood.
4. Dehydrate slides in graded ethanol (70 %, 80 %, 90 %, 100 % ethanol, each for 1 min), (*see* **Note 3**).
5. Air-dry slides in an oven at 50 °C.
6. Apply 10 μL of HER2/chromosome 17 probe mixture to a 22 × 22 mm slip.
7. Invert slides and gently lower them onto 22 × 22 mm slips with probe mixture.
8. Seal slides with rubber cement.
9. Hybridize slides overnight at 37 °C, shielded from light (*see* **Note 12**).
10. Remove rubber cement, place slides in 0.5× SSC buffer at room temperature to make the cover slip fall off.
11. Place slides into 0.5× SSC buffer at 72 °C for 2 min.

12. Air-dry slides in an oven shielded from light at 50 °C.
13. Mount slide in mountant with 0.2 ng/mL DAPI and seal with resin glue (*see* **Note 13**).
14. Carefully remove excess glue with a soft paper, and ready to evaluate the slide by microscopy.

3.3 Chromogenic In Situ Hybridization

All procedures in this section should be performed after completed Basic Sample Handling procedures.

1. Apply 10 μL probe solution (Zymed Spot-Light series) and seal the slides under UnderCover Slips (*see* **Note 14**).
2. Denature probe and target DNA, 94 °C for 5 min (*see* **Note 15**).
3. Hybridize slides overnight at 37 °C, shielded from light (*see* **Note 12**).
4. Remove UnderCover Slips.
5. Wash slides with 0.5× SSC buffer for 5 min at 75 °C.
6. Wash slides with PBS buffer, 5 min.
7. Incubate slides for 45 min at room temperature, using mouse antidigoxigenin antibody (diluted 1: 500 in the Powervision + blocking solution).
8. Wash slides with PBS buffer, 5 min.
9. Incubate slides for 20 min at room temperature, using Powervision + post antibody blocking solution (*see* **Note 16**).
10. Wash slides with PBS buffer, 5 min.
11. Incubate slides for 30 min at room temperature, using Powervision + poly-HRP-goat-x-mouse polymer (*see* **Note 16**).
12. Wash slides with PBS buffer, 5 min.
13. Incubate slides for 5 min at room temperature, using the Powervision + DAB solution (1000 μL distilled water + 50 μL DAB reagent A + 50 μL DAB reagent B, prepared ready for use).
14. Wash slides with distilled water, 1–2 min.
15. Stain slides with standard hematoxylin, 1–5 s.
16. Wash slides with distilled water, 5–10 s.
17. Dehydrate slides in graded ethanol (70 %, 80 %, 90 %, 100 % ethanol, each for 1 min), (*see* **Note 3**).
18. Immerse slides in xylene for 10 min, twice.
19. Mount slides in 0.2 ng/mL DAPI.
20. Cover with a cover slip and seal with resin glue.
21. Carefully remove excess glue with a soft paper, and ready to evaluate the slide by microscopy.

4 Notes

1. Filters specific for DAPI and different spectrums are required and a 100× objective application was preferred.
2. Regularly, both internal and external controls are required for FISH. For large-scale sample scanning, both control sections should be included within each diagnostic run.
3. It is best to prepare this series of graded ethanol fresh each time before use. When used for 10 batches of slides, it is also best to replace them with fresh ones.
4. Sodium thiocyanate is used to break the protein–protein disulfide bonds, which could facilitate the subsequent process of digestion.
5. Fresh pepsin should be added to digestion buffers before starting digestion, and additional pepsin should also be added each 30 min during the whole process of digestion.
6. Even though nearly no attenuation of FISH signals was found when stored for prolonged periods, use of slides within 4 months of cutting is strongly preferred.
7. A fume hood should be used when handling xylene. Some nonorganic solutions (Hemo-de solution for example) may be used as an alternative.
8. As a pretreatment permeabilization process, the use of 0.2 N HCl to acid deproteination allows preservation of better tissue morphology.
9. After washing slides in wash buffer, gently touch the slide edge by absorbent paper to remove remaining fluid.
10. This step is very crucial to achieve clear images, because exposure of slides to protease buffer could ensure adequate pretreatment of tissues prior to application of probes.
11. If digestion of tissue is optimal, proceed to next steps. Otherwise, proceed to **steps 17** and **18** and then repeat the protease treating steps for 1–15 min digestion depending on the detailed condition (*see* **step 11–16**).
12. To ensure the hybridization efficiency, duration of this step should be more than 12 h.
13. Transparent resin glue could be used to prevent slides from drying out.
14. Conventional repeat-containing DNA probes also work well in CISH, as an alternative to Zymed probes.
15. Denaturation of probe and the target DNA is carried out shortly after applying the probes on slides.
16. Probe detection is crucial in all CISH steps. To increase sensitivity, a two-layer antibody approach was applied.

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