Interreg Italia-Österreich
precanmed

SOP –UNITS-4.0
Author:
E. Campaner,
A. Zannini
Approved: **G. Del Sal**
Issued:
12/04/2019
Revised:

HUMAN MAMMARY ORGANOID CHARACTERIZATION - IMMUNOHISTOCHEMISTY

Purpose

The SOP-UNITS-4.0 was issued to describe how to perform histological characterization of human mammary organoids.

Scope

SOP 4.0 is intended to cover all resources, personnel and equipment needed to perform histological characterization of human mammary organoids.

Introduction

Histological characterization of human mammary organoids by immunohistochemistry is fundamental to ensure that organoids resemble the histological characteristics of the original tissue.

Personnel at UNITS prepare organoids for immunoistochemistry analyses and collaborators at *Anatomia Patologica Unit of the Cattinara Hospital* perform a routine histopathological analysis on organoids.

1. Cell culture media, reagents and solutions

- dH_2O
- PBS 1X (Life Technologies, cat. no. 14190-094)
- Cell recovery solution (Corning cat. no. 354253)
- Paraformaldehyde (Sigma-Aldrich, cat. no. 158127)
  !CAUTION Paraformaldehyde contains formaldehyde, which can cause cancer; handle it using appropriate safety gear.
- FBS (Euroclone cat. no. ECS0180L)
- Tris Buffered Saline with Tween® 20 (TBST-10X, Cell Signaling cat. no. 9997)

- Tris base (Sigma-Aldrich, cat. no. T1503)
- Primary antibodies for IHC
- Agarose (Sigma-Aldrich, cat. no. A5093)
- CDI's Tissue Marking Dye (Cancer Diagnostics cat. no. 0728-3)
- Xylene (Sigma-Aldrich, cat. no.247642)
- Ethanol anhydrous denatured (Sigma-Aldrich, cat. no.51976), histological grade (100% and 95%)
- Hydrogen peroxide (Sigma-Aldrich, cat. no. 216763)
- Hematoxylin (Sigma-Aldrich, cat. no. H3136)
- Eosin Y (Sigma-Aldrich, cat. no. 230251)
- SignalStain Antibody Diluent (Cell Signaling, cat. no. 8112)
- Citrate (Sigma-Aldrich, cat. no.1613859)
- EDTA (Sigma-Aldrich, cat. no.1233508)
- Pepsin (Sigma-Aldrich, cat. no.P0525000)
- SignalStain® Boost IHC Detection Reagents (Cell Signaling, cat no. 8125 and 8114)
- SignalStain® DAB Substrate Kit (Cell Signaling, cat. no 8059)
- Mounting medium

2. Equipment

- High-speed centrifuge (Eppendorf centrifuge 5810R)
- Pipette aid, serological pipettes (Euroclone cat no. EPS05N; EPS10N)
- Pipettes
- Pipette tips
- Conical centrifuge tubes (Euroclone, cat. no. ET5015B; ET5050B)
- Microcentrifuge tubes (Euroclone, cat. no. ET3415)
- Microscopes slides Superfrost® Plus (Thermo Scientific, cat. no. J1800AMNZ)
- Staining jars
- PAP Pen for immunostaining (Sigma-Aldrich, cat. no. Z377821)
- Coverslips
- Microscope
- Ice

3. Reagent setup

- 1% Agarose in H2O: to dissolve agarose, boil the solution in the microwave. If required, add a minimal amount of CDI's Tissue Marking Dye. This will render agarose block clearly visible in the paraffin wax.
- Wash buffer (1X TBST). To prepare 1 L of 1X TBST: add 100mL 10X TBST to 900mL dH2O and mix
- 10 mM Sodium Citrate Buffer: To prepare 1 L, add 2.94 g sodium citrate trisodium salt dihydrate to 1 L dH2O. Adjust to pH 6.0
- 1 mM EDTA: to prepare 1 L, add 0.372 g EDTA to 1 L dH2O. Adjust to pH 8.0
- TE: 10 mM Tris/1 mM EDTA, pH 9.0: To prepare 1 L, add 1.21 g Tris base and 0.372 g EDTA to 950mL dH2O. Adjust pH to 9.0, then adjust volume to 1 L with dH2O
- Pepsin solution: 1 mg/ml in Tris-HCl, pH 2.0
- 3% hydrogen peroxide solution: to prepare 100mL, add 10mL 30% hydrogen peroxide to 90mL H2O
- Blocking Solution: TBST/5% FBS. To 4.75mL 1X TBST, add 250µL FBS
- Primary antibodies solution: dilute primary antibodies at an assay depending concentration in SignalStain Antibody Diluent

4. Procedure: immunohistochemistry of breast organoids

4.1 Sample preparation

4.1.1 Grow organoids for 5-7 days following procedure described in SOP2 Human breast organoid cultivation. Organoids derived from 3-6 wells of a 24-well plates are generally sufficient to perform immunohistochemistry analysis. !CRITICAL! For the following steps precoat all the tubes, the pipettes and tips with FBS to avoid attachment of the tissue to the plastic.

4.1.2 Keep the plates on ice. Remove the medium from each 24 well, add 500µL of cold PBS to wash organoids. Aspirate PBS and add 500µL of cold PBS.

4.1.3 Using p1000 pipette tips displace Matrigel from the wells and collect the material (PBS and organoids in Matrigel) in a 15ml tube and centrifuge it at 200g for 5 minutes at 4˚C.

4.1.4 Remove the supernatant and add Cell Recovery solution (~100µL each well). Incubate organoids in this solution for 1 hr at 4˚C in order to gentle digest Matrigel and release cells with minimal cell damage. Organoids will settle under gravity.

Carefully aspirate the supernatant.

4.1.5 Add 3mL of cold PBS and let organoids settle under gravity.

4.1.6 Carefully aspirate the supernatant.

4.1.7 Fix organoids by adding 3mL of 4% Paraformaldehyde for 20 min at RT (shaking slowly). Organoids will settle under gravity.

4.1.8 Carefully aspirate the supernatant and add 3mL of PBS and let organoids settle under gravity. Repeat this step.

4.1.9 Cut off and discard the conical bottom of 1.5mL microcentrifuge tube and cap the tube.

4.1.10 Carefully aspirate the supernatant and resuspend organoids pellet in 200µL of 1% agarose pre-dissolved in microwave using a cut tip. Transfer them in the cap of the previously prepared microcentrifuge tube (the cylinder of agarose will be prepared in the cap of the tube) and put the tube immediately on ice. This step allows the embedding of organoids in agarose blocks, which can be easily embedded in paraffin wax.

4.1.11 Extract the agar cylinder from its base and process them into paraffin blocks using standard tissue processing.

4.1.12 From paraffin blocks, cut thin slices (4-6 µm) on a microtome, and float sections in a water bath.

4.1.13 Mount sections on to charged slides and dry overnight. Using charged slides helps the section to adhere to the slide.

4.2 Deparaffinization/Rehydration

To perform immunohistochemistry, paraffin wax must be removed from the sample and the sample must be rehydrated !CRITICAL! Do not allow slides to dry at any time during this procedure as this can lead to inconsistent staining.

4.2.1 To remove paraffin wax, place sections in xylene containing jars for three times for 5 minutes each. Fresh xylene should be used as incomplete deparaffinization can also lead to inconsistent staining.

4.2.2 To start rehydration, place sections in 100% ethanol containing jars for two times for 10 minutes each.

4.2.3 Place sections in 95% ethanol containing jars for two times for 10 minutes each.

4.2.4 To complete the rehydration process, wash sections two times in dH2O for 5 minutes each.

4.3 Antigen Unmasking

Tissue pre-treatment with the antigen retrieval reagents breaks the protein cross-links formed by formalin fixation and thereby allows the uncover of hidden antigenic sites. !CRITICAL! Consult product datasheet for antibody-specific recommendation for the unmasking solution.

For Citrate: Bring slides to a boil in 10 mM sodium citrate buffer, pH 6.0; maintain at just below boiling point for 10 minutes. Cool slides at RT for 30 minutes.

For EDTA: Bring slides to a boil in 1 mM EDTA, pH 8.0; maintain at just below boiling point for 10 minutes. Cool slides at RT for 30 minutes.

For TE: Bring slides to a boil in 10 mM Tris/1 mM EDTA, pH 9.0 (See 4.1.1 and 4.1.2); maintain at just below boiling point for 18 minutes. Cool at room temperature for 30 minutes.

For Pepsin: Digest for 10 minutes at 37 °C.

4.4 Staining

This protocol is for chromogenic staining. !CAUTION Consult product datasheet for recommended antibody diluent.

4.4.1 Wash sections in dH2O three times for 5 minutes each using staining jars.

4.4.2 To quench endogenous peroxidase activity in samples, which may lead to high background staining, place sections in 3% hydrogen peroxide for 10 minutes.

4.4.3 Wash sections in dH_2O two times for 5 minutes each using staining jars.

4.4.4 Wash sections in wash buffer for 5 minutes.

4.4.5 Use the PAP Pen to make an hydrophobic barriers on glass slides around the sample, taking care not to touch the sample. This allows to use small amounts of reagents in the following steps.

4.4.6 To prevent non-specific binding of the antibody to the tissues, block each section with few drops of blocking solution for 1 hour at RT in a humidified chamber.

4.4.7 Remove blocking solution and add 100–400µL of primary antibody solution to each section. Incubate overnight at 4˚C in a humidified chamber

4.4.8 Equilibrate SignalStain Boost Detection Reagent to room temperature.

4.4.9 Remove antibody solution and wash sections in wash buffer three times for 5 minutes each using staining jars.

4.4.10 Cover sections with 1–3 drops SignalStain Boost Detection Reagent as needed. Incubate in a humidified chamber for 30 minutes at RT.

4.4.11 Wash sections three times with wash buffer for 5 minutes each using staining jars.

4.4.12 Add 1 drop (30µL) SignalStain DAB Chromogen Concentrate to 1mL SignalStain DAB Diluent and mix well before use.

4.4.13 Apply 100–400µL SignalStain DAB to each section and monitor closely until a brown reaction product is yielded. 1–10 minutes generally provides an acceptable staining intensity.

4.4.14 Immerse slides in dH_2O.

4.4.15 For better visualization of tissue morphology, counterstain sections with hematoxylin. This reagent has a deep blue-purple colour which stains the cell nuclei and provides a contrast to the brown reaction product of the DAB chromogen.

4.4.16 Wash sections in dH_2O two times for 5 minutes each.

4.5 Dehydrating and Mounting Sections

SignalStain DAB Chromogen is compatible with either aqueous or non-aqueous mounting medium. If choosing non-aqueous, the sections must be dehydrated again prior to coverslip mounting.

4.5.1 Place sections in 95% ethanol containing jars for two times for 10 minutes each.

4.5.2 Place sections in 100% ethanol containing jars for two times for 10 minutes each.

4.5.3 Place sections in xylene containing jars for two times for 5 minutes each.

4.5.4 Mount sections with coverslips using mounting medium, being careful to avoid introducing air bubbles.

4.5.5 Allow mounting medium to set and view slides on a microscope (***Figure 1***).

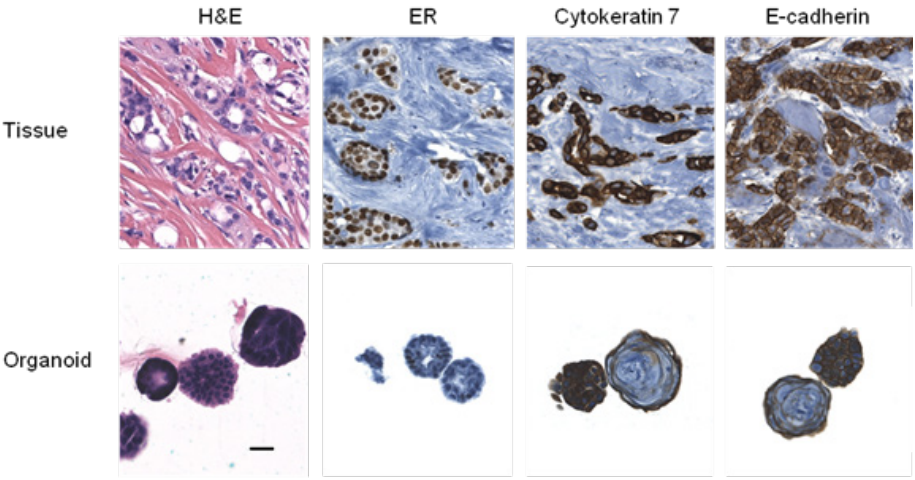

Figure 1. Organoids recapitulate histological features of the original tumor. Representative images of Hematoxylin/Eosin (H&E) and immunohistochemistry stainings on sections of a Luminal A invasive ductal breast carcinoma (top panels) and of the corresponding tumor organoids (bottom panels). Immunohistochemistry was performed using antibodies raised against Estrogen Receptor (ER), Cytokeratin 7 and E-cadherin. Scale bar 10 µm.

5. Applicable references to SOPs

SOP-UNITS-2.0

6. References

- Sachs, et al., A living biobank of breast cancer organoids captures disease heterogeneity. Cell 10.1016/j.cell.2017.11.010 (2017)