BAYLOR COLLEGE OF MEDICINE
BREAST CENTER
Houston, Texas

Estrogen Receptor, (6F11)

Important Procedural Notes:

All steps are performed at room temperature unless otherwise noted.
See "General Reagents" section for details on preparation and storage.

The protocol may be performed with or with out Sequenza Coverplates (ThermoShandon) and racks, however it is the laboratory's standard to use the coverplates from steps 14-18 with the following changes:

Rinsing with TBS-20 stated as, "Rinse x 3 and hold in TBS-20" is to be substituted by completely filling each coverplate-slide well with TBS-20 and allowing the wells to drain. Only the final 80-100uL of TBS-20 should remain and no buffer should be visible from the top of the well, prior to the application of the next reagent.

All antibody steps will be performed by using a 200 uL standard amount per incubation.

Initial Slide Preparation

1. Cut sections at 3-4 microns onto **Fisher PLUS slides** (best slides in our experience).
2. Adhere tissue by baking overnight (10-12 hrs) at 58 ^{o}C in a *dry* slide incubator.
3. Deparaffinize in graded alcohols and xylene in a routine manner:

   a. The slides are to be deparaffinized on the Shandon-Lipshaw Varistain using Program 2. Press *LOAD* and hang the basket above Station 1 (Xylene 1). Press *LOAD* again to lower the basket into the Xylene. Press *RECALL* > *2* > *ENTER* > *RESET* > *AUTO* to start staining. Be sure that agitation is turned on during the process. The programmed steps are as follows:

STATION	REAGENTS	PROGRAM 2
1	XYLENE	5 MIN
2	XYLENE	5 MIN
3	XYLENE	5 MIN
4	100% ETHANOL	3 MIN
5	100% ETHANOL	3 MIN
6	100% ETHANOL	2 MIN
7	95% ETHANOL	3 MIN
8	WATER	1 MIN
	END	

   b. When the deparaffinization run is complete, the stainer alarm will sound. Slides are removed from the stainer (station 8) by pressing the following sequence of buttons: *AUTO*> *LOAD* then remove slides from the stainer then press LOAD again to close the stainer lid.
4. Place slides upright in Tissue-Tek plastic slide baskets (VWR) in NPW and transfer to **TBS-20**.

Estrogen Receptor, (6F11)

Antigen Retrieval (AR)

5. Fill 24-slide capacity Tissue-Tek staining dishes (the green heat-resistance plastic composite type) with **Tris-HCl 9 AR buffer**.
6. Place the buffer-filled dishes in a 5-quart pressure cooker containing about 1.7 liters of deionized water in the bottom (the water is there to create super-heated steam).
7. With the pressure cooker lid off, preheat the AR buffer to 90°C using an "IR 6100 Infrared Hot Plate" (Fisher, 11-493-615) at 70% power (preheating takes about 10 minutes). Cover the dishes to reduce evaporation.
8. Immerse the slides in racks into the preheated AR buffer and close the pressure cooker lid with the pressure-control valve setting at "2" (based on the T-FAL OPTIMA pressure cooker).
9. Continue heating until full pressure is achieved (wait for hissing sound at about 3 minutes).
10. Heat for another 10 minutes at full pressure -*timing here is critical*.
11. Immediately open the pressure-release valve, depressurize fully (about 2 minutes), and remove the lid of the pressure cooker.
12. Remove the slides in racks from the pressure cooker, rinse in six changes of nanopure water (NPW), and hold in TBS-20.

Blocking

13. Block endogenous peroxidase by immersing slides in **3% hydrogen peroxide solution** in a coplin jar for 5 minutes. Rinse in 3 changes of NPW and hold in TBS-20 for 5-10 minutes.
14. Remove from TBS-20 quickly to avoid drying and block endogenous biotin by covering tissue on prone slides with 3-4 drops of **Avidin A solution** (A/B Blocking Kit by Vector Laboratories) and incubating for 15 minutes. Rinse x 3 and hold in TBS-20.
15. Remove from TBS-20 quickly to avoid drying and cover tissue on prone slides with 3-4 drops of **Biotin B solution** (A/B Blocking Kit by Vector Laboratories) and incubate for another 15 minutes. Rinse x 3 and hold in TBS-20.

Primary Antibody

16. Remove from TBS-20, quickly to avoid drying, and cover tissue on prone test slides with 3-4 drops of **ER (6F11),** Novocastra diluted at **1:200** in antibody diluent. Incubate for 1 hour at room temperature**.** Negative control slides are incubated with **"non-immune" antibody** (Dako) matched for species, type, isotype, and concentration. Rinse x 3 and hold in TBS-20.

Linking Antibody and Enzyme

17. Remove from TBS-20, quickly to avoid drying, and cover tissue on prone slides with 3-4 drops of **MIgG-Biotin (Rabbit) Dako at 1:200** in antibody diluent and incubate for 30 minutes. Rinse x 3 and hold in TBS-20.
18. Remove from TBS-20, quickly to avoid drying, and cover tissue on prone slides with 3-4 drops of *FRESHLY* prepared **LSA (HRP) Dako** diluted at **1:200** in TBS-20 and incubate for 30 minutes. Rinse x 3 and hold in TBS-20.

If cover plates are being used, remove at this time and perform one more rinse in TBS-20 before proceeding

Estrogen Receptor, (6F11)

Chromogen

19. Remove from TBS-20, quickly to avoid drying, and cover tissue on prone slides with 3-4 drops of **DAB+ solution** (Dako) and incubate for 15 minutes *(CARCINOGEN = use appropriate precautions).* Rinse x 6 and hold in NPW.
20. Under a fume hood, wipe away excess NPW from each slide and enhance the chromogen signal by applying 3-4 drops of **0.2% Osmium tetroxide** (Sigma) on prone slides. Incubate for 30 seconds *(TOXIC = use appropriate precautions).* Rinse x 6 and hold in NPW.

Counterstain and Coverslip

21. Counterstain slides in **Harris Hematoxylin** (Thermo-Shandon) for 30 seconds and dehydrate and clear in a routine manner:

    a. The slides are to be counterstained, dehydrated and cleared on the Shandon-Lipshaw Varistain using **Program 3**. Press *LOAD* and hang the basket above **Station 9** (95% ETOH #2 after Eosin). Press *LOAD* again to lower the basket into the 95% ETOH. Press *RECALL* > *1* > *ENTER* > ***STEP until station 9 is reached*** > *AUTO* to start counterstaining. Be sure that agitation is turned on during the process. The programmed steps are as follows:

STATION	REAGENT	PROGRAM 1
9	HARRIS ACIDIFIED HEMATOXYLIN	30 SEC
10	WATER	1 MIN
11	0.25% ACID ALCOHOL (in 70% ETOH)	15 SEC
12	WATER	1 MIN
13	AMMONIA WATER (0.75mL/700mL DH20)	1 MIN
14	WATER	1 MIN
15	95% ETHANOL	1 MIN
16	EOSIN	45 SEC
17	95% ETHANOL	15 SEC
18	95% ETHANOL	15 SEC
19	100% ETHANOL	30 SEC
20	100% ETHANOL	30 SEC
21	100% ETHANOL	1 MIN
22	XYLENE	1 MIN
23	XYLENE	30 SEC
24	XYLENE	30 SEC
25	END	END

Estrogen Receptor, (6F11)

b. When the counterstaining run is complete, the stainer alarm will sound. Slides are removed from the stainer (station 24) by pressing the following sequence of buttons: *AUTO> LOAD* then remove slides from the stainer then press LOAD again to close the stainer lid.

23. Mount sections in Permaslip (Stat-Lab) with appropriately sized coverslips (24mm x 40mm are appropriate for most specimens).

Interpretation

Staining is seen as a dark brown to black nuclear signal in cells expressing ER (e.g. 70-80% of invasive breast cancers).

GENERAL REFERENCES:

1. Allred DC, Harvey JM, Berardo M, Clark GM : Prognostic and predictive factors in breast cancer by immunohistochemical analysis. Modern Pathol 11(2):155-168, 1998.

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4. Mohsin SK, Bardou V-J, Arpino G, Chamness GC, Allred DC. Prognostic and predictive factors in breast cancer: An evidence-based medicine approach. In: Nabholtz J-M, Tonkin K, Reese DM, Aapro MS, Buzdar AU. ed. Breast cancer management: Application of clinical and translational evidence to patient care (2nd Edition), Lippincott Williams & Wilkins, Hagerstown, MD. 391-422, 2003.

SPECIFIC REFERENCES VALIDATING THIS TECHNOLOGY:

1. Harvey JM, Clark GM, Hilsenbeck SG, Osborne CK, Allred DC: Immunohistochemistry is superior to ligand binding assay for evaluating estrogen receptor status in a study of 1,982 breast cancer patients. J Clin Oncol 17:1474-1481, 1999.

2. Elledge RM, Green S, Pugh R, Allred DC, Clark GM, Hill J, Ravdin P, Martino S, Osborne CK: ER and PgR by ligand binding assay compared with ER, PgR, and pS2 by immunohistochemistry in predicting response to tamoxifen in metastatic breast cancer: A Southwest Oncology Group Study. Int J Cancer 89:111-117, 2000.

3. Ellis MJ, Coop A, Singh B, Mauriac L, Llombert-Cussac A, Janicke F, et al. Letrozole is more effective neoadjuvant endocrine therapy than tamoxifen for ErbB-1- and/or ErbB-2-positive, estrogen receptor-positive primary breast cancer: evidence from a phase III randomized trial. J Clin Oncol 19(18):3808-16, 2001

4. Love RR, Duc N, Allred DC, Binh NC, Dinh NC, Kha NN, Thuan TV, Mohsin SK, Roanh LD, Khang HX, et al. Oophorectomy and tamoxifen adjuvant therapy in premenopausal Vietnamese and Chinese women with operable breast cancer. J Clin Oncol 20(10):2559-66, 2002