

- **TITLE: Immunohistochemistry & IF Procedure Manual.**

- **CONTENT:**

A. IHC PROCEDURE MANUAL (including QA&QC)

B. Direct Immunofluorescence

C. VENTANA IMMUNOSTAINER

A. IMMUNOHISTOCHEMISTRY PROCEDURE MANUAL

1. DEFINITION :

1.1. Immunohistochemistry is the localization of antigens or proteins in tissue sections using a labeled antibody as specific reagent through antigen-antibody interactions that are visualized by a marker such as chromogenic dye.

1.2. Antibody: An immunoglobulin capable of specific binding with the antigen.

1.3. Monoclonal: A homogeneous population of antibodies that are raised by the fusion of B cells with immortal cell cultures to produce hybridomas.

1.4. Polyclonal: Multiple B cell response to an antigen resulting in a mixture of antibodies typically recognizing a variety of epitopes on the antigen.

2. POLICY:

2.1. All persons working in the Immunohistochemistry are responsible for maintaining QC documentation and ensuring compliance with established QA procedures.

2.2. The QC officer is responsible for overseeing these activities.

2.3. When staining frozen sections, use a protocol that does not include hydrogen peroxide. Hydrogen peroxide can cause bubbling, which in turn affects staining.

2.4. Positive and negative tissue controls are stained for each antibody marker, and should be processed and fixed in the same manner as the patient specimen.

2.5. The positive and negative tissue control is attached at the slide lower part or on a separate slide (Batch control) for the specific

antibody, ensuring that all steps in both control and specimen are performed the same way.

2.6. Immunohistochemistry worksheet HIST-FRM 010 and slides are sent to the pathologist for evaluation.

2.7. PH of buffers is checked and adjusted whenever prepared and routinely monitored and documented in Log Book.

2.8. All antibodies used in histopathology section of King Fahd Hospital of the university, are labeled “**for in vitro diagnostic use**”.

2.9. All new Immunohistochemistry antibodies, prior to use, are validated against multiple known positive and negative tissue controls. (FRM-HIST 033)

2.10. When new lot numbers are received, evaluate against tissues stained with the old lot Numbers. (Parallel test). Document on (FRM-HIST 036).

3. PROCEDURE :

3.1. SPECIMEN REQUIREMENTS

3.1.1. Paraffin embedded sections including decalcified tissue and alcohol –fixed cell block.

3.1.2. Cryostat sections (for DIF).

3.1.3. Cytological preparations (Cytopins, smears).

3.2. SPECIMEN STORAGE

3.2.1. Paraffin sections: at room temperature.

3.2.2. Cryostat sections: ... Store at - 70°C (up to 6 months). Before using bring slides to room temperature.

3.3. SUPPLIES AND MATERIALS

3.3.1. Ventana Ultra advanced automated stainer (See operation manual)

3.3.2. Frosted positively charged slides.

3.3.3. Cover slips.

3.3.4. Positive and Negative Tissue Controls.

3.3.5. Light Microscope.

3.3.6. Deionized Distilled Water.

3.3.7. Mounting medium.

3.3.8. REAGENTS:

- DAB detection kit from Ventana.
- Amplification kit.

- Protease-1.
- Bluing reagent.
- Hematoxylin Counter stain.
- Cell conditioning 1 (CC1).
- Cell conditioning 2 (CC2).
- EZ prep from Ventana.
- Reaction buffer from Ventana.
- Liquid coverslip (High temperature).
- Sodium Chloride Sodium Citrate (SSC).

3.4. PREPERATION OF THE SLIDES.

3.4.1. Cytopathology slides

- 1) Immediately after preparation, before drying fix in 95% ethanol for 10-15 minutes at room temperature then air-dry.
- 2) Immerse the slides in Phosphate Buffer Saline for 5 – 10 minutes.

3.4.2. Cryostat Sections

- 1) On cryostat cut 4 micron- sections.
- 2) Collect specimens on clean-coated slides.
- 3) Dry slides at room temperature for 2 hours or overnight.
- 4) If necessary to stain section at the same day they are cut, sections may be fixed in cold acetone (2 – 8°C).
- 5) In case of stored slides (slides that cut before and stored for 3 – 6 months), prior to stain, bring them to room temperature.
- 6) Fix in acetone at room temperature for 10 minutes then air dry. Proceed with Immunostaining.

3.4.3. Formalin Fixed Paraffin-Embedded Sections

- 1) **Two-4** micron thick sections.
- 2) The specimen is mounted on the upper part of the slide and tissue controls on the lower part of the slide, Or on a separate slide.
- 3) Slides baking: incubate the slides in oven at temperature 50 – 60 °C, for 15 – 20 min or overnight at 37°C.

3.5. RESULTS

3.5.1. A positive reaction appears golden brown to dark brown at the sites of specific cellular antigen.

3.5.2. A negative reaction appears like a Hematoxylin counterstain.

3.6. HER-2, ER/PR test by IHC:

3.6.1. Fixation guidelines:

- 1) Specimens should be immersed in 10% neutral buffered formalin within 1 hour of the biopsy or resection procedure
- 2) If delivery of a resection specimen to the lab is delayed, the tumor should be bisected prior to immersion in fixative (the surgeon ensures that the identity of the resection margins is retained in the bisected specimen, alternatively, the margins may be separately submitted).
- 3) The time of removal of the tissue and the time of immersion of the tissue in fixative should be recorded and submitted to the laboratory.
- 4) Specimen should be fixed for 6-72 hours. **If fixation time exceeds that range, this should be documented in the patient report.**
- 5) Formalin volume should be at least 10 times the volume of the specimen.

3.6.2. Her2, ER, PR assay validation: Initial test validation included 20 positive and 20 negative cases (FDA-approved Ventana tests). Validation performed by comparing our laboratory's results with Specialist Hospital assay that has been appropriately validated. Concordance levels were 95%. Equivocal cases were not included in Her2 validation.

In case of significant changes in testing methods (e.g. antibody clones, antigen retrieval protocol or detection system), revalidation is required.

3.6.3. At least one tumor sample from all patients with breast cancer (early-stage, recurrent, or metastatic disease) is tested for **ER\PR, HER2** protein expression (IHC assay).

3.6.4. Report elements for IHC tests of **Her2, ER, PR**, should include:

- 1) Type of specimen fixation and processing.
- 2) Antibody clone and general form of detection system.
- 3) Scoring system

3.6.5. ER, PR, Her2 REPORTING AND INTERPRETATION IS DONE ACCORDING TO ASCO/CAP GUIDELINES. THE PATIENT REPORT SHOULD INCLUDE WHICH METHOD OF SCORING IS USED (ASCO/CAP REFERENCE, WITH THE YEAR OF PUBLICATION).

3.6.6. Estrogen (ER) and Progesterone (PR) receptors SCORING:

1) Scoring Criteria:

- Positive test means staining of equal or greater than 1.0 % of tumor cells.
 - Negative test means staining of less than 1.0 % of tumor cells.
- #### 2) Positive staining interpretation is estimation the percentage of staining over the tumor present. (See percentage estimation below).
- #### 3) Tumor estimation criteria:
- 3+ > 50% staining of tumor cell nuclei.
 - 2+ = 25 – 50% staining of tumor cell nuclei.
 - 1+ > 1.0% staining of tumor cell nuclei.
- #### 4) Staining is reported as indeterminate if there are problems in handling, and processing the sample.

3.6.7. HER-2 NEU SCORING CRITERIA.

1) Her-2 Protein Scoring System:

- Score 0 Negative: No staining or faint membrane staining of less than 10% of tumor cells.
- Score 1+ Negative: Weak incomplete membrane staining in more than 10% of tumor cells.
- Score 2+ Weak Positive: Weak/moderate (“thin”) **complete** membrane staining in more than 10% of tumor cells.
- Score 3+ Strong Positive: Strong (thick) complete membrane staining in more than 10% of tumor cells.

3.6.8. Cases reported (Her2, IHC) equivocal (2+) should be tested by (ISH).

3.6.9. HER2 testing should be repeated on another specimen or block if:

- 1) The initial HER2 Result is discordant with the histologic features of tumor
- 2) In a core biopsy, the initial result is negative when the amount of tumor used for testing was limited, or
- 3) The result is **equivocal** by IHC and ISH.

3.6.10. ANNUAL RESULTS COMPARISON FOR ER AND PR:

For IHC tests that provide independent predictive information, the laboratory at least annually compares its patient results with published benchmarks, and evaluates inter-observer variability among the pathologists in the laboratory.

NOTE: Individuals interpreting the assay must also have their concordance compared with each other and this concordance should also be at least 95%.

With specific reference to estrogen and progesterone receptor studies: in general, the overall proportion of ER-negative breast cancers (invasive and DCIS) should not exceed 30%. The average is somewhat lower in postmenopausal than premenopausal

women (approximately 20% vs. 35%).

The average is considerably lower in well-differentiated carcinomas (<10%) and certain special types of invasive carcinomas (<10% in lobular, tubular, and mucinous types). The proportion of PgR-negative cases is 10-15% higher than for ER in each of these settings. Investigation is warranted if the proportion of negative cases is significantly higher in any of these settings.

3.7. LIMITATION OF IMMUNOHISTOCHEMISTRY (IHC).

3.7.1. Tissue staining is dependent on a proper fixation and processing. Improper fixation, freezing, thawing, washing, drying, heating, sectioning, or contamination with other tissues or fluids may produce artifacts, antibody trapping, or false-negative results.

3.7.2. Excessive or incomplete counter-staining may compromise proper interpretation of Results.

3.7.3. Use of old or other fixative than neutral buffered formalin, or exposure of tissues to excessive heat (greater than 60°C) during processing may result in decreased staining sensitivity.

3.7.4. Normal non-immune sera from an animal source as the secondary antisera used in blocking steps may cause false-negative or false positive results due to autoantibodies or natural antibodies.

3.7.5. Immunohistochemistry does not replace Hematoxylin and Eosin stain, but is the complement to it and should never be used alone for any diagnostic purposes.

3.7.6. Immunohistochemistry is a multiple step diagnostic process that requires specialized training in the selection of appropriate reagents, tissue selections, fixation, processing, preparation of the Immunohistochemistry slides, and interpretation of the staining results.

3.7.7. Inconsistent results may result from variations in fixation and embedding methods, or from inherent irregularities within the tissue.

3.8. IHC QUALITY CONTROL

3.8.1. For IHC staining, pathologist should evaluate the negative and positive controls prior to evaluate patient samples. Results of controls must be documented on (FRM-HIST 023) before reporting patient results.

3.8.2. Positive and Negative Tissue Controls.

Each antibody test requires an appropriate positive and negative tissue control that assess the performance and specificity of the primary antibody and serves as an indicator that specimen processing and staining were carried out correctly. The positive control must contain the target antigen. The same tissue control serves as positive and negative control (**Internal negative control is acceptable**). Whenever possible, controls prepared in-house must be used. (See ventana.com for recommended positive controls)

3.8.3. When batch controls are run, the pathologist on-duty reviews all control slides of patient testing that day and document its results on FRM-HIST 010. This control review records are retained for 2 years.

3.8.4. The batch control slides location is maintained at IHC unit for review.

3.8.5. After verifying the controls a statement “**All controls show appropriate reactivity**” is included in the patient report with the IHC results.

3.8.6. Negative Reagent Control: Our IHC protocol use polymer-based detection system (biotin-free), so negative **Reagent** control is not required.

3.9. ANTIBODY EVALUATION AND VALIDATION:

3.9.1. Almost all the antibodies used in KFHU histolab are (ready to use) from VENTANA which are validated and optimized by the manufacture.

3.9.2. For evaluation of these antibodies, use 2 positive and one negative tissue controls. Results should be approved by a pathologist and documented in the antibodies validation form (FRM-HIST 033).

3.9.3. New tissue block control must be evaluated with known antibody on old block (parallel test).

3.9.4. Multi-tumor block is usually used to test the range of antibody staining.

3.9.5. For **concentrated antibodies**, dilution testing must be done to determine optimum dilutions. The technologist who performs the test must check each final optimized dilution and document the results on the Optimal Dilution sheet.

1) New concentrated antibodies should be tested at dilutions above and below the manufacturers recommended dilutions. Tests are continued until the optimum dilution is found.

2) Re-ordered antibodies (same lot) dilution is determined from the previous lots. If necessary, the optimal dilution is revised and entered on the Optimal Dilution sheet.

3) If necessary, the antibody may be tested on a variety of normal and tumor tissue types chosen from references in the product information sheet received with each antibody and approved by a pathologist.

4) The optimized dilution test slide should be stored in the Optimal Dilution slide box with the name of the Antibody, Lot Number, Optimal Dilution, Date and Control Tissue noted on the slide.

3.10. IHC ASSAY PERFORMANCE.

Confirm assay performance with at least 10 known positive and 10 known negative cases when an existing validated assay has changed in any of the following ways:

1) Antibody dilution, antibody vendor (same clone), or the incubation or retrieval times (same method).

2) Confirm assay performance by testing a sufficient number of cases (defined by head) to ensure that assays consistently achieve expected results when any of the following have changed: fixative type, antigen retrieval method (e.g. change in pH, different buffer, different heat platform), antigen detection system, tissue processing or testing equipment, environmental conditions of testing (e.g. laboratory relocation), or laboratory water supply.

3) Document on LAB.MED-FRM-HIST 023

3.11. New lots of antibody and detection system reagents performance:

3.11.1. New lots of reagents are compared with old lots before or concurrently with being placed into service.

3.11.2. Parallel staining of the new lots with the old lot is required for comparison using an appropriate panel of control tissues (slides cut from the same tissue control block).

3.11.3. Document results on new lot verification form (HIST-FRM 036).

3.12. ANTIBODY STORAGE.

3.12.1. Antibodies must be stored properly to ensure their shelf life is as long as possible. Temperatures of the refrigerators and freezers must be monitored. The -70°C freezers should have an alarm set-up to warn of power loss or a rise in temperature.

3.12.2. Refrigerator: Antibodies may be stored in the refrigerator at $2 - 8^{\circ}\text{C}$ if recommended by the manufacturer or if the diluent contains sodium azide or other anti-bacterial agent.

3.12.3. Freezer -70°C : Antibodies may be aliquoted and stored in this freezer if recommended by the manufacturer. This freezer should not be a frost-free freezer as the temperature cycling may damage the antibodies.

3.13. REAGENT PREPERATION.

3.13.1. All reagents used in immunohistochemistry must be meticulously prepared.

3.13.2. Standard formulas, which have been tested in this laboratory and entered in the procedure manual, are to be adhered to at all times. Testing of new formulations will be done as required on control tissues.

3.13.3. The pH of the buffers used should be checked and if required, adjusted for each batch. (Reaction buffer - $\text{pH } 7.6 \pm 0.2$)

3.13.4. The pH meter must be calibrated on the day it is used. Enter the pH testing records in the logbook to keep records of pH calibration.

3.13.5. Dates of preparation and expiration must be noted on each bottle, as well as the initials of the person who prepared the reagent.

3.13.6. Unless indicated otherwise on the container, all reagents should be stored at room temperature.

3.14. TISSUE PROCESSING AND PARAFFIN SECTIONING.

3.14.1. For accurate and reproducible results, tissue must be processed according to established guidelines in the histology procedure manual. Every care should be taken to process tissue optimally as under or over fixation will result in variable staining or, in extreme cases, no staining at all. Furthermore, exposure to temperatures higher than 60°C during processing and slide drying should be avoided as this may cause damage to antigens. Paraffin sections for immunohistochemistry are to be cut at 2-4µm and mounted on positively charged slides (SuperFrost “Plus”). Sections must be of superior quality.

3.15. CONTROL TISSUE SELECTION AND STORAGE.

3.15.1. Selection of Control Tissue is determined by consulting the product information sheet and a consultant pathologist. The control tissue used should be processed “in-house” in order to prevent any variations due to differences in fixation procedures in different laboratories.

3.15.2. Frozen tissue blocks and slides should be stored at -70°C. Slides can be stored at 4°C for a week if previously fixed in acetone.

3.15.3. Paraffin embedded tissue chosen for controls are stored in the IHC working area for quick access. The tissue may be sectioned, mounted on coated slides and stored in slide boxes until used. The tissue block should be stored with the slides. Additionally, the slides should be labeled with the original surgical number to assure association with a specific case.

3.15.4. A reference slide of each control block can be stored with the cut slides and tissue block.

3.16. ASSURING TEST CONSISTENCY.

3.16.1. Immunohistochemical stains are useful only if the established procedures give consistent results from day to day and between different technologists. The following steps are crucial in producing consistently good results:

1) **Antibody Dilution:** Each laboratory must test to find the optimal dilution for every antibody they use. After this is established, good, clean staining with consistency should be the aim. New batches of the same antibody should also be checked for proper staining.

2) **Incubation Time:** Incubation time should be kept constant. This is of great importance, especially for a “Day-run” as variations in staining time will affect the intensity of the staining and/or background staining.

3) **Temperature:** Unless otherwise indicated all incubations for manual immunohistochemical staining techniques are performed at

room temperature (some procedures use 37°C incubation in order to shorten the incubation times).

4) Controls: Control tissue slides are used to assure the specificity of the stain. They also act to assess the overall quality of the staining. A positive control tissue is used to confirm the specificity of the staining, and a Negative control slide will highlight any non-specific staining that may occur, endogenous peroxidase staining for example.

3.17. DOCUMENTATION.

3.17.1. Dilution Testing: An Optimal Dilution Form for each concentrated primary antibody is to be filled out and signed by the senior technologist and a consultant pathologist. The slide demonstrating the optimal dilution will be kept in the Optimal Dilution slide Box.

3.17.2. Expired Antibody Testing: If necessary, an expired antibody or reagent may be used for teaching purpose only (not for diagnosis) if it is still found to be of acceptable quality (see Product Certification for specific guidelines).

3.17.3. Control Tissue in Use: Control tissue must be tested and then assessed by a pathologist. A Control Tissue form must be filled out and signed by the Technologist and a Pathologist. A slide showing proper staining must be placed in the box of cut control slides along with the control tissue block. Frozen tissue blocks and slides are kept in the -70 °C freezers and a slide showing the proper staining is kept in the Frozen Tissue Control slide box.

3.17.4. Daily Review: The technologist will review the daily output of slides and remedy any problems noted. The Pathologist on duty has to review all positive and negative controls on a daily basis, and document his findings/comments.

B. Automated Direct Immunofluorescence (DIF).

1. PURPOSE :

1.1. To establish a policy for a standardized use and maintenance of Ventana Automated stainer for Direct Immunofluorescence (DIF) staining on frozen section.

2. DEFINITION :

2.1. Direct immunofluorescence staining is a specific type of immunohistochemistry, usually to detect the presence of immunoglobulin and complement in the skin and kidney. A batch of fluorescein isothiocyanate-labelled antibodies against IgG, IgM, IgA, fibrin and C₃, C₄ is applied to frozen sections of fresh tissue and examined by fluorescence microscopy.

2.2. Fluorescent: Molecular label on antibody that emits a distinct, measurable color spectrum in response to a specific laser or chemical interaction.

2.3. CLINICAL SIGNIFICANCE.

Immunofluorescence is used in conjunction with a panel of antibodies to aid in the identification of immunoglobulin in target cells and tissues (e.g. in the diagnosis of renal and/or skin pathology). Characteristic staining patterns are seen in the immunobullous disease, lupus erythematosus and vasculitis.

NOTE: The clinical interpretation of any staining, or its absence, must be complemented by morphological studies and evaluation of proper controls. Evaluation must be made by a qualified pathologist within the context of the patient's clinical history as well as other diagnostic tests.

3. POLICY :

3.1. If DIF staining is required, medical technologists in the histology laboratory IHC area will prepare the frozen section block.

3.2. All persons performing direct immunofluorescent staining techniques must follow the method outlined below. Stained slides must be stored in slides folders in the refrigerator IHC area at 4°C. Stains are viable up to 1 week although the staining intensity does deteriorate over time.

3.3. The technologists in the IHC area will check the DIF Worksheet weekly (form FRM-HIST 022) and discard into sharps containers, the slides that have already been interpreted and the staining results documented.

4. PROCEDURE :

4.1. SAFETY.

4.1.1. All specimens should be handled with care as if they are infectious.

4.1.2. Always wear gloves.

4.1.3. Because knives are extremely sharp, extra caution is advised.

4.1.4. Acetone is highly flammable. Keep it away from heat.

4.1.5. For more safety information, refer to MSDS.

4.2. POSITIVE TISSUE CONTROL.

4.2.1. Control tissue should be fresh biopsy or surgical specimen frozen as soon as possible in a manner identical to test section. Positive staining cell /tissue component (e.g. germinal centers/ lymphocyte) are used to confirm that FITC antibodies are correctly applied and the instrument function properly, not as an aid in formulating a specific diagnosis of patients sample.

4.3. NEGATIVE TISSUE CONTROL.

4.3.1. The same tissue (e.g. tonsil or lymph node) used for the positive tissue control may be used as the negative tissue control. The

non-staining component (e.g. the tonsillar mantle zone) should demonstrate absence of specific staining.

4.3.2. Internal antigens serve as positive control (e.g. IgA in tubular casts, IgG in protein droplets and C₃ in blood vessels). When internal positive controls are absent, external positive controls are required. Non-reactive elements in the patient specimen may serve as a negative tissue control.

4.4. SPECIMEN COLLECTION.

4.4.1. Prompt and rapid freezing of the specimen as soon as possible after it is removed from the patient is necessary for the proper freezing of tissue, “cut 5 micron and picked on” positively charged slides.

4.4.2. The slide should be fixed in an acetone bath for no more than 2 to 5 minutes. Unstained slides should then be stored for no more than 10 days prior to use.

4.5. EQUIPMENTS.

4.5.1. Ventana Ultra operation Manual.

4.5.2. Fluorescence microscope.

4.6. CONSUMABLES.

4.6.1. Reaction Buffer (dilute prior to use 10X).

4.6.2. Liquid Cover Slip LCS (ready to use).

4.6.3. Acetone (follow safety guidelines).

4.6.4. Aqueous mounting media.

4.7. REAGENTS.

- FITC C₃.
- FITC C₄.
- FITC Fibrinogen.
- FITC IgA.
- FITC IgG.
- FITC IgM.

Note: All FITC Antibodies are ready to use from FDA approved Ventana.

4.8. SLIDE PREPERATION.

4.8.1. Cut 4 micron thick sections on a super frost plus slide.

4.8.2. Stain the first section with H&E to confirm:

-In the case of renal biopsies – there are glomeruli present.

-In the case of skin biopsies – there is dermis and epidermis present.

-If not, deeper levels are required to obtain a satisfactory section, otherwise, Immunofluorescence will not be useful.

4.8.3. Air dry slides for 30 minutes or store slide at – 70⁰C to be stained later.

4.8.4. Fix slides in acetone for 10 minutes at room temperature.

4.8.5. Air-dry for 10-20 minutes.

4.9. LOADING SLIDES AND REAGENT.

4.9.1. For more details on operation of Ventana Ultra refer to next section.

4.9.2. The FITC antibody vial and desired accessory reagent are loaded into the reagent tray, and placed on the Ventana automated slide stainer.

4.9.3. Double check the slide labeling. Each slide must be labeled with appropriate bar code specifying the staining procedure.

4.9.4. Load the frozen section slides.

4.9.5. Apply sufficient amount of reaction buffer solution to wet the tissue on each slide then press run.

4.10. THE FOLLOWING IS THE SEQUENCE OF EVENT CARRIED OUT BY THE VENTANA AUTOMATED STAINER.

4.10.1. The slide is rinsed with solution.

4.10.2. Liquid coverslip is applied to the slide.

4.10.3. The optimized FITC is applied and incubated with mixing for 4 to 32 minutes at 42^oC.

4.10.4. The slide is rinsed with wash solution.

4.11. POST-RUN STEPS.

4.11.1. Wash slides in distilled water, dehydrate in ethanol, and Keep the slides in the dark for 10 minutes.

- 4.11.2. Mount in aqueous mounting medium.
- 4.11.3. Circle location of tissue on the back of the slide with a marking pen.
- 4.11.4. Place the slide in slide tray and keep in the refrigerator.
- 4.11.5. Register the DIF case on (FRM-HIST-022) and inform the pathologist.

NOTES: Efficient removal of LCS from the slides following removal from the instrument will greatly reduce background autofluorescence. To accomplish this, rinse slide thoroughly in reaction buffer solution. The slide may then be rinsed in distilled water and cover slipped using aqueous base mounting media. Slide should be read within one day of staining, stable up to 3 days if properly stored at 2-8 °C.

4.12. RESULTS.

4.12.1. A positive staining is indicated by a bright apple green staining. The location and the pattern of staining are used to diagnose the disease process and photographs may be taken for a permanent record and results of the Test and Control should be documented on FRM-HIST-022 and in the patient's final report.

C. Ventana Ultra Advanced Staining System for Immunohistochemistry.

1. DEFINITION :

1.1. The BenchMark ULTRA Advanced Staining System is intended to automatically stain Histological or cytological specimens on microscope slides with specific Immunohistochemistry (IHC) or in situ hybridization reagents for in vitro diagnostic use. Evolved from the BenchMark series of instruments, the BenchMark ULTRA instrument fully automates the processes of baking, deparaffinization, and staining.

1.2. Accession, run, and report the staining process and manage reagents, staining protocols, users, and slide cases.

1.3. The ability to define and store protocols for IHC staining ensures reliable, high quality results.

1.4. Protocols are defined step by step and saved on the computer. When IHC staining is required, protocols can be printed on a slide bar code label. The staining instrument reads the protocol from the bar code and applies the defined detection procedure and staining process.

1.5. Faster turnaround time of routine and complex staining.

1.6. The BenchMark ULTRA system also expands and enhances functions that support slide processing, including automating protocol handling, label printing, orders, inventory, case management, reagent registration, reporting, and system maintenance.

2. POLICY :

2.1. 'Ventana Ultra advanced staining system' must have been read and understood by users prior to use the instrument. All persons using the Ventana Ultra advanced staining system must be reasonably conversant with its use and maintenance and must undergo extensive training under the guidance of the senior medical technologist or other experienced operators before using the instrument on their own. All persons are responsible for performing routine maintenance.

2.2. No changes to programs or procedures are permitted without the express permission of the Histopathology supervisor or designee. Expiry dates of reagents that are not monitored by the Ventana Medical systems software must be checked regularly to ensure that they are still valid. The instrument and its environment must be kept clean at all times.

2.3. Any instrument failures must be reported to the Histopathology supervisor/Designee.

2.4. Any solutions prepared / reconstituted must document the pH on solution preparation log book.

3. PROCEDURE :

3.1. SAFETY

3.1.1. All safety related regulations, local codes, and instructions that appear in the manual or on equipment must be observed to ensure personal safety and to prevent damage to the instrument or equipment connected to it. If equipment is used in a manner not specified by the manufacturer, the protection provided by the equipment may be impaired.

3.1.2. Some of the reagents used in immunohistochemistry are hazardous. Be aware of local regulations and correct procedures for handling and disposing of hazardous material. When working with any reagent or reagent container, take appropriate precautions.

3.1.3. Avoid unnecessary contact with reagents and reagent containers.

3.1.4. Always wear approved eye protection, gloves, and protective clothing when you handle reagents, reagent containers, slide trays, or preparation trays.

3.2. QUALITY CONTROL

3.2.1. The system uses barcode labels to identify individual slides and track staining protocols. All staining procedures must be quality controlled using a known positive control.

3.2.2. The control must be run simultaneously with the test under exactly the same conditions.

3.2.3. The completed stain must be checked microscopically and the results of the control must be documented in the IHC result sheet and signed by the pathologist.

3.2.4. All maintenance and preventative maintenance must be documented on the daily maintenance checklist.

3.3. REAGENTS AND CONSUMABLES :

3.3.1. **EZ Prep Concentrate (10X)** - EZ Prep Concentrate (10X) solution (EZ Prep) is used for paraffin removal from tissue samples during immunohistochemistry. This reagent is intended for *in vitro* diagnostic (IVD) use. Do the following to prepare diluted (1X) solution.

3.3.1.1. Check the spigot to ensure it is in the off position before adding water to the carboy.

3.3.1.2. Fill a graduated 20 L carboy approximately three quarters full with distilled or deionized water.

3.3.1.3. Pour the entire contents of the 2 L EZ Prep Concentrate (10X) bottle into the carboy.

3.3.1.4. Fill the carboy to the 20 L graduation mark with distilled or deionized water. If large quantities of bubbles form during the filling procedure, allow the solution to sit until the bubbles have dissipated.

3.3.1.5. Place the cap on the container, mix the solution well to ensure that the concentrate is thoroughly mixed with the water. The diluted EZ Prep (1X) solution is ready to use.

3.3.2. **Liquid Coverslip (LCS)** - Non-reactive light oil that is used during the slide staining process on the staining module to prevent evaporation.

3.3.3. **Cell Conditioning1 (CC1)** – Cell Conditioning Solution (CC1) is a pre diluted solution used as a pretreatment step in the processing of tissue samples for IHC. CC1 is a tris based buffer which must not be diluted. Fixation of tissue by formalin results in the formation of covalent bonds between the aldehyde and amino groups present in the tissue. The formation of these bonds denatures protein and can result in the loss of antigenicity. In addition, the formaldehyde can form methylene bridges cross linking tissue proteins thus reducing the penetration of the tissue to large molecules such as antibodies. CC1 is a tris based buffer with a slightly basic pH, which, at elevated temperatures is capable of hydrolyzing the covalent bonds formed by formalin in tissue. Removing these bonds allows renaturation of protein molecules and increases antibody accessibility. Often these changes result in significant gains in antibody binding and improved signal to noise ratios. The automated slide stainer automatically heats the slide to the appropriate temperature and time as selected by the user.

3.3.4. **Reaction Buffer (10X)** - Reaction Buffer Concentrate (10X) is a Tris based buffer Solution (pH 7.6 ± 0.2) used to rinse slides between staining steps and provide a stable aqueous environment for the immunohistochemistry (IHC). Ventana Reaction Buffer is used as a key component in maintaining a proper aqueous environment for many reactions to occur during IHC and ISH, such as general washing, antibody incubation and incubation of enzymes and other ancillaries when used on the automated slide stainer. The IHC reaction involves a specific antibody that is localized by a biotin conjugated secondary antibody formulation that recognizes rabbit and mouse immunoglobins. This step is followed by the addition of an avidin/streptavidin enzyme conjugate that

binds to the biotin present on the secondary antibody. The specific antibody, secondary antibody streptavidin, enzyme complex is then visualized with a precipitating enzyme reaction product. Each step is incubated for a precise time and temperature. At the end of each incubation step, the Ventana automated slide stainer washes the sections with Reaction Buffer to stop the reaction and remove unbound material that would hinder the desired reaction in subsequent steps. To make the diluted solution from the 2 L bottle of Reaction Buffer Concentrate (10X):

- a. Be sure the spigot of the 20 L graduated carboy (supplied with the automated slide stainer) is in the OFF position before filling.
- b. Fill the empty 20 L carboy approximately 75% full with deionized or distilled water.
- c. Pour contents of 2 L Reaction Buffer Concentrate (10X) bottle into the water in the carboy. Swirl to mix
- d. Fill carboy to the 20 L fill line with deionized or distilled water. While adding the water, swirl the solution to ensure that the concentrate becomes fully mixed. If large quantities of bubbles occur during the filling step, allow the wash solution to settle. Once bubbles have settled, add the remaining volume of water necessary to make 20 L of wash solution.
- e. Loosely screw the cap on the carboy. If cap is too tight, the solution will not feed properly from the spigot. The wash solution is now ready to use with the Ventana automated slide stainer.
- f. Measure the PH Buffer after preparation

3.3.5. UltraView Universal DAB Detection Kit - UltraView Universal DAB Detection Kit is an indirect, biotin-free system for detecting mouse IgG, mouse IgM and rabbit primary antibodies. The kit is intended to identify targets by immunohistochemistry (IHC) in sections of formalin-fixed, paraffin-embedded and frozen tissue that are stained on the BenchMark ULTRA stainer. This product is intended for *in vitro* diagnostic (IVD) use. The *ultraView* Universal DAB Detection Kit detects specific mouse and rabbit primary antibodies bound to an antigen in paraffin-embedded tissue sections. The specific antibody is located by a cocktail of enzyme labeled secondary antibodies. The complex is then visualized with hydrogen peroxide substrate and 3, 3'-diaminobenzidine tetrahydrochloride (DAB) chromogen, which produces a brown precipitate that is readily observed by light microscopy.

Warning: Possible carcinogen. The International Agency for Research on Cancer (IARC) and the US National Toxicology Program (NTP) have listed benzidine, a compound closely related to 3, 3'-diaminobenzidine tetrahydrochloride (DAB), as a known human carcinogen.

3.3.6. Hematoxylin Counter stain - Hematoxylin counterstain reagent is a modified Gill's hematoxylin and is intended for staining cellular nuclei on slides containing cells from frozen tissue, formalin fixed and paraffin embedded tissue, or cytologic preparations.

3.3.7. Bluing Reagent / Post counter stain - Bluing Reagent works through the combined effect of lithium ions and raising the pH of the wash buffer to blue the hematoxylin stained sections. 1 Bluing Reagent is applied after hematoxylin and changes the hue of the hematoxylin to a blue color.

3.3.8. **Protease 1** - Protease 1 is an endopeptidase (alkaline protease) of the serine protease family and cleaves antigens (proteins) in the tissue section, allowing primary antibodies to recognize and bind epitope(s). The reagent is intended for enzymatic digestion of sections of routine formalin-fixed, paraffin-embedded tissue on VENTANA automated slide stainers. Protease 1 provides consistent results for antigens requiring extensive digestion for optimal staining.

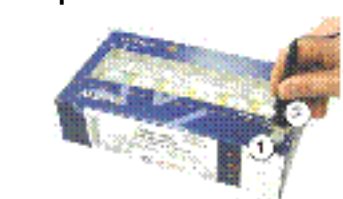
3.4. SPECIMEN:

- 3.4.1. Paraffin embedded tissue fixed in 10% neutral buffered formalin.
- 3.4.2. Decalcified tissue fixed in 10% neutral buffered formalin.
- 3.4.3. Cryostat sections.
- 3.4.4. Cytological preparations (cystospins, smears).
- 3.4.5. Cut paraffin sections at 2-4um on positively charged super frost glass slide.

3.5. OPERATING INSTRUCTIONS :

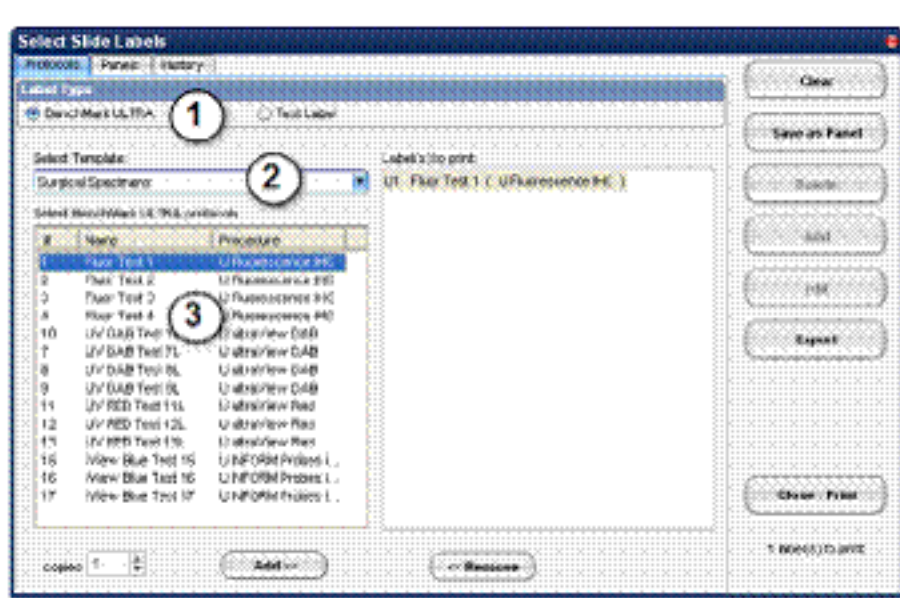
3.5.1. **Product Registration:** All Ventana reagents, antibodies, and bulk products are packaged with a memory button (1) attached to the container. The product registration probe is attached to the computer. When you touch the probe (2) to the memory button, it reads the product information and transfers it to the computer. All the information you need to use the product is entered automatically and accurately.

- a. On the main menu top right side click to “Display inventory view”
- b. Select “Wand” and scan the button provided on to the ventana product.
- c. The computer reads data from the button and confirms for Registration.

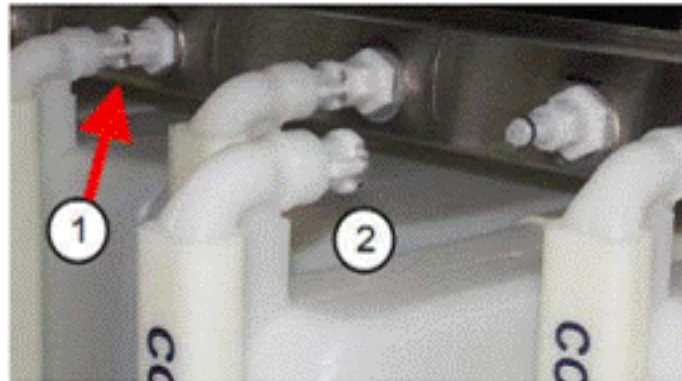


3.5.2. Printing barcode labels:

- a. At the Home View, click the Create Label button. SLS opens (fig.1)
- b. Click the Protocols button. The Select Slide Labels options appear (fig.2)
- c. Select the label template and protocols (fig.3)



3.5.3. Pre-Run Preparation:



a. Refill bulk bottle.

b. Empty the waste module if more than three quarters full.

To remove a bulk bottle, press the latch release (1) and pull the bottle out (2).

To install a bulk bottle:

1. Refill the bottle from the carboy.
2. Place the bottle in the slot and push it forward until you feel or hear it click.

1. Press the Lock button to unlock the waste container, and then pull it out. The waste container monitor on the front of the instrument turns red.
2. Empty the container into the appropriate waste disposal container.
3. Push the container back until it locks into place. The waste container monitor on the front of the instrument turns off.



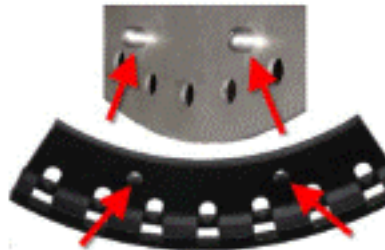
Figure 18. Waste Container Lock

CAUTION: Handle waste containers carefully. To avoid injury, wheel the carboy to the disposal area. To prevent spillage, put the cap on the carboy before removing it.

- c. Record the pH of Reaction Buffer, EZ prep and other buffer whenever prepared.

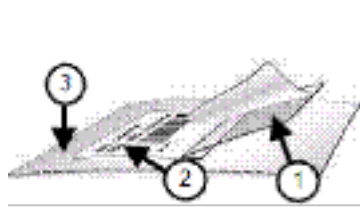
3.5.4. Starting a Run:

- 3.5.4.1. Before starting the run always Wash the slide pads by selecting washing option.
- 3.5.4.2. Install a reagent tray.
 - a. Select the required reagents
 - b. Make sure the dispensers are snapped into the holders on the tray.
 - c. Lift the tray from the magnetic tray holder.
 - d. Slip the mounting holes in the reagent tray onto the mounting studs on the carousel.



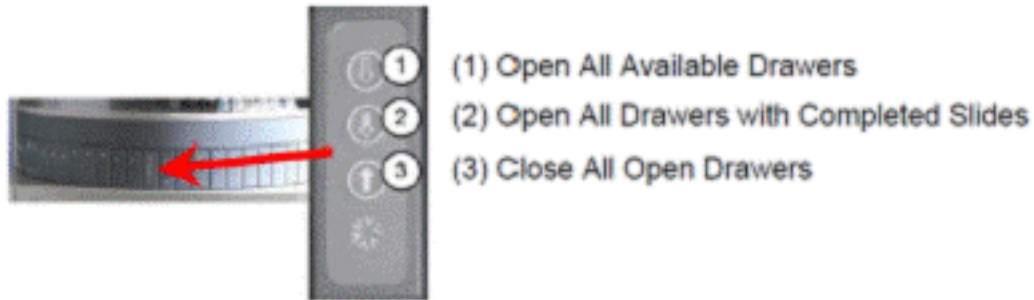
- 3.5.4.3. Apply slide labels

- a. After the label is printed, tear it off carefully.
- b. Push the transparent flap (1) over the label (2) to seal the printed side.
- c. Peel the label from the backing (3).
- d. Apply the label to the frosted side of the slide.

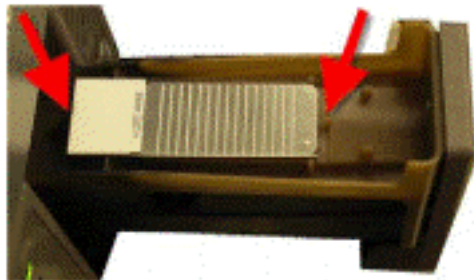


3.5.4.4. Load Slides

- a. At the Instrument View, make sure the instrument is in Ready mode. If necessary, click the Ready button. When the instrument is ready to use, the slide drawer lights will turn green.
- b. Press the Open All Available Drawers button on the instrument Slide Control Panel.



- c. Position slides in drawers with labels facing up and inward. Wiggle each slide and push it down gently to make sure it is seated on the heating pad. Both ends of the slide are held in place by small posts.
- d. Press the Close All Open Drawers button on the instrument Slide Control Panel.



3.5.4.5. Make sure the reagent hood and all drawers are closed.

3.5.4.6. At the Instrument View, click the Running mode button.

3.5.4.7. The system reads the reagent and slide bar codes, calculates the staining run time, and starts the run. During the run, the Instrument Bar shows the countdown to run completion.

3.5.5. Unload Completed Slides: When slides are completed, their images in the Instrument View and their drawer lights on the instrument flash green.

a. Press the Open All Drawers with Completed Slides button on the instrument Slide Control panel.

b. Remove the slides from the open drawers.

c. Press the Close All Open Drawers button on the instrument Slide Control Panel.

3.5.6. After Run:

a. After you remove the slides from the instrument, rinse them in warm soapy water to remove any remaining reagents.

b. Dehydrate the slides as usual and apply a glass coverslip to each slide.

3.5.7. Adding Slides while slides are Running- Once the instrument is in Running mode, you can add slides without disrupting slides that are already running. (Any required reagents must already be on the reagent carousel or you will need to schedule a landing zone to load them.) Available drawers are indicated by green lights. Whenever a drawer opens and closes, its light flashes yellow. The system automatically scans it to look for a slide and incorporate it into the run. Each slide is processed independently and has its own Run Report.

3.5.7.1. To add a single Slide.

a. Locate an available drawer (the green light is lit).

b. Press the Drawer Control button on the instrument or the Open button in the software.

c. Load the slide and close the drawer. When staining begins, the yellow light stops flashing.

3.5.7.2. To add group of slides.

a. Press the Open All Available Drawers button.

b. Load the slides into the drawers you want to use.

c. Press the Close All Open Drawers button. When staining begins, the yellow lights stop flashing.

Important: The drawers can be open and close individually, but processing starts sooner if you close all the drawers at once.

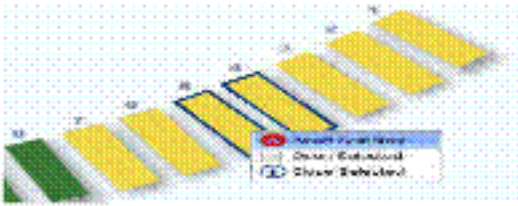
3.5.8. Cancel Slides:

a. Click the instrument name in the Instrument Bar.

b. At the Instrument View, click the image of the slide you want to cancel. To select more than one slide, CTRL click each image.

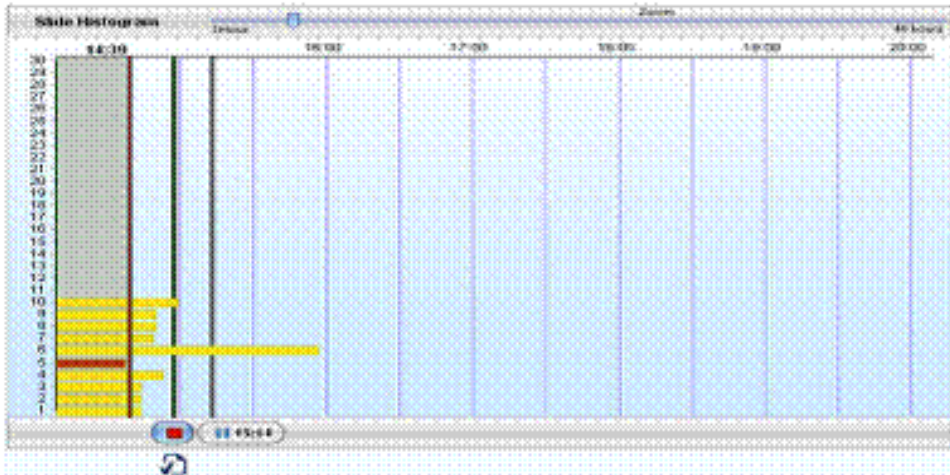
Selected drawers are outlined.

- c. Right click any selected drawer, and then click Abort Selected.



3.5.9. Slide Processing Time:

- a. Click the instrument name in the Instrument Bar.
- b. At the Instrument View, click the Slide button at the left of the screen. The Slide Histogram opens.
- c. Find the slide number in the histogram. Each horizontal bar represents a slide drawer. The bar length corresponds to the slide processing time. The vertical lines to the right of the Now marker indicate possible landing zones.
- d. Stop the mouse cursor on a bar to see popup information. Double click a bar to open the Order Details view. Popup information and Order Details are the same as in View Slide Details, above.



3.5.10. Landing Zones - The BenchMark ULTRA system won't start a staining run unless the reagents loaded are sufficient to complete the run. But because the system continuously processes slides, the requirements to complete all loaded slides can change after the initial run begins. It might occasionally happen that the reagents loaded aren't sufficient to process both the original batch and the added slides. In that event, the system must pause so the reagent hood can be opened to add or change dispensers. A pause is accomplished by scheduling a landing zone. A landing zone is a system-calculated time at which processing can pause without jeopardizing any slide in the staining run. Whether or not a landing zone is available depends on the current protocol and processing

steps. You select a landing zone on the computer. The software then directs the instrument to pause at the specified time and alerts you when the time arrives.

3.5.10.1. Schedule a landing zone - When a run requires reagents that are not on the carousel, status indicators turn red and error messages appear. Check the Slide or Reagent Histogram to see if landing zones are available.

- a. Click the instrument name in the Instrument Bar to open the Instrument View.
- b. At the Instrument View, click the Slide or Reagent button at the left of the screen. The Histogram opens. Possible landing zones are indicated by vertical lines. Beneath each vertical line is a Pause button that shows the time of the associated landing zone.
- c. Click a Pause button. The vertical line turns green. The Pause button becomes a red Stop button, and a Pick List button appears below it.
- d. Click a Pause button. The vertical line turns green. The Pause button becomes a red Stop button, and a Pick List button appears below it. While the instrument is in the landing zone, the indicator in the Instrument Status Panel lights up. Until the required reagents are actually loaded onto the instrument, status indicators for the affected slides remain red.



Landing Zone Indicator on the Instrument

3.5.10.2. Landing zone progress - The histogram below shows the interval between the time a landing zone is selected and the time it starts. The hatch marks on each slide bar show the interval between the time that slide pauses and the time the landing zone starts. After you select a landing zone, the time to completion shown in the histogram, the Instrument Bar and the Home View is adjusted to include the time the slide is paused. If the time exceeds what is efficient for laboratory work flow, you can click the landing zone button again to cancel the landing zone and resume slide processing.

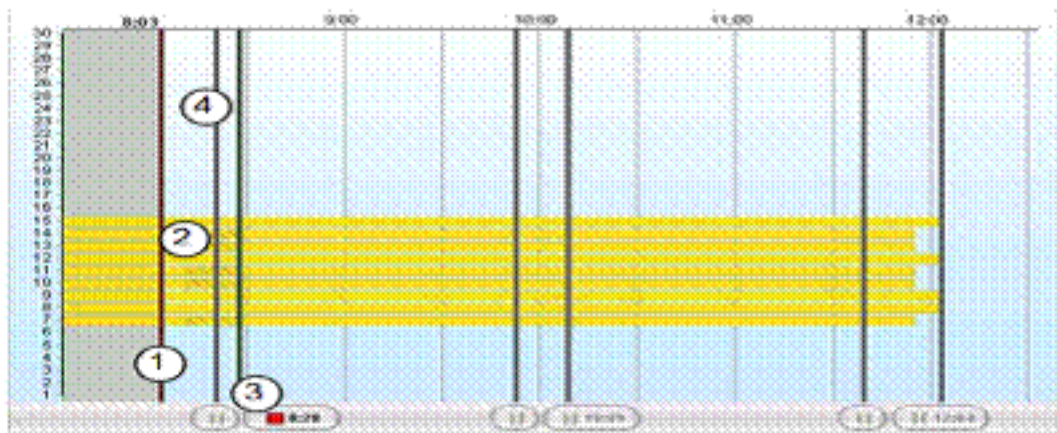


Figure 50. Landing Zone Processing Delays

Feature	Description
1 Current time	The red vertical bar shows the current time.
2 Hatched slide bar	The hatch-marked section of the slide bar indicates the length of time the slide is paused with the selected landing zone. Rest the cursor on the hatch marks to see a popup message that shows the slide number and the exact length of the delay.
3 Landing zone time	The button on the right of the landing zone bar shows the time the zone will begin.
4 Optional landing zone	A gray vertical line with a gray pause button indicates a landing zone that is not selected.

3.5.10.3. During a Landing Zone - All you need to do is open the reagent hood, load or remove reagents, and close the hood. When the hood closes, the processing resumes automatically. The hood must be opened and closed within a defined time period. At the start of the landing zone, the system alerts the operator and displays messages in the Communicator. A 5-minute countdown is displayed in the Instrument Bar. If the hood hasn't been opened when the countdown ends, the system will resume processing. If the reagent hood is opened within 5 minutes, a 10-minute countdown appears in the Instrument Bar. If the hood is still open when the countdown ends, the system alerts the operator and displays messages prompting to finish loading and close the hood. When the hood is closed, the system automatically resumes processing. If the hood is not closed within 10 minutes, warning messages will be displayed. If too much time goes by, the entire run will be terminated.

3.5.10.4. Cancel a landing zone:

- a. Click the instrument name in the Instrument Bar.
- b. At the Instrument View, click the Slide or Reagent button at the left of the screen. The Histogram opens. The selected landing zone is indicated by a red Stop button and the countdown to the scheduled time.

c. Click the red Stop button. It becomes a blue Pause button, and the landing zone is no longer active.

3.5.11. Maintenance Checklist:

Task	Daily	Monthly	Quarterly
Wipe External Surfaces	X		
Pre-Run Check List	X		
Clean slide drawers		As needed	
Rinse Bulk Fluid Containers		X	
Wash and Brush Slide Heating Pads		X	
Clean Waste Tub and Drain		X	
Run Slide Heater Temperature Verifiers			X
Decontaminate Bulk Fluid Bottles			X
Decontaminate Bulk Fluid Reservoirs			X
Decontaminate the Instrument		Done by Ventana technicians	

Note: For more details on operation and troubleshooting an electronic copy available on the desktop of the instrument. For quality control and Quality assurance of Immunohistochemistry refer to (IHC QC&QA)

RESPONSIBILITY:

All Histopathology staff.

ATTACHMENTS :

Lab.Med.FRM-HIST 022 Results log of Immunofluorescence.

Lab.Med.FRM-HIST 010 IHC daily QC review.

Lab.Med.FRM-HIST 033 Antibody Validation.

Lab.Med.FRM-HIST 036 New Lot Verification.

DITRIBUTION:

- LMD Administration Office
- Histopathology Laboratory Section

- DQSA.

REFERENCES :

- Ventana Operation Manual and Antibody Inserts.
- Hammond MEH, Hayes DF, Dawsett M, et al. American society of Clinical Oncology/ College of American Pathologists guideline recommendation for Immunohistochemical testing of estrogen and progesterone receptors in breast cancer. Arch Pathol Lab Medicine 2010; 134:907-922.
- College of American Pathologist (CAP) Checklist – 2014