Title:	EMT Panel IFA Staining for Tumor Biopsy Slides				Page 1 of 26
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Please check for revision status at

https://dctd.cancer.gov/ResearchResources/ResearchResources-biomarkers.htm

and be sure to use the current version.

Change History

Revision	Approval Date	Description	Originator	Approval
--	8/03/2017	New Document	KFG/DK	JJ
A	6/16/2020	Updated SOP340549 to SOP340550 throughout the document; Updated critical reagent and software information; minor editorial changes throughout the document.	KFG/LL	

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OVERVIEW OF IMMUNOFLUORESCENCE ASSAY FOR BIOPSIES

SOP340507:

Tumor Frozen Needle Biopsy Specimen Collection and Handling

- Collect and freeze tumor needle biopsies for use in biomarker assays

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SOP340550:

Tumor Frozen Needle Biopsy Preparation for Pharmacodynamic Immunofluorescence Assays Utilizing Murine Testis and/or Jejunum Control Tissues

- NBF fix and paraffin embed tumor needle biopsies and control tissues
- Section biopsies for use in EMT IFA
- Stain slides by H&E for standard histology analysis

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SOP340546:

EMT Panel IFA Staining for Tumor Biopsy Slides

- Load biopsy and control slides into Bond-RX Processing Module
- Bond-RX automated staining of slides with EMT Panel Critical Reagents
- Stain slides with DAPI and mount cover slips

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Image within 72 h

SOP340547:

Whole Slide Image Capture of Tumor Biopsy Slides of EMT Panel IFA

- Capture images of EMT Panel IFA stained biopsy slides and control slides using Aperio ScanScope FL

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SOP340548:

Image Extraction and Analysis of Tumor Biopsy Slides from EMT Panel IFA

- Quantitate captured images of EMT Panel IFA-stained biopsy slides and control slides using ImageScope and Definiens Tissue Studio analysis software

1.0 PURPOSE

To standardize an immunohistochemical method for detecting and quantifying an epithelial-mesenchymal transition (EMT) panel of markers for evaluating the phenotype of human tissue biopsies within formalin-fixed, paraffin-embedded tissue sections to support various pharmacodynamic (PD) studies. The EMT Panel includes E-cadherin, Vimentin and β-Catenin for tumor segmentation. The goal of the SOP and associated training is to ensure consistency of EMT Panel IFA measurements.

2.0 SCOPE

This procedure applies to all personnel involved in the use of the EMT Panel Immunofluorescence Assay (IFA) for tumor biopsies from patients participating in clinical trials. This SOP outlines the recommended procedure for staining of paraffin-embedded tumor biopsy sections using the automated Leica Bond-RX™ Automatic Staining System.

3.0 ABBREVIATIONS

Ab	=	Antibody
AF488	=	Alexa Fluor® 488
AF546	=	Alexa Fluor® 546
AF647	=	Alexa Fluor® 647
DAPI	=	4',6-Diamidino-2-Phenylindole
DCTD	=	Division of Cancer Treatment and Diagnosis
DI	=	Deionized
ER	=	Epitope Retrieval
H&E	=	Hematoxylin and Eosin
HIER	=	Heat-Induced Epitope Retrieval
EMT	=	Epithelial to mesenchymal transition
ID	=	Identification/Identifier
IFA	=	Immunofluorescence Assay
LHTP	=	Laboratory of Human Toxicology & Pharmacology
NA	=	Numerical Aperture
PADIS	=	Pharmacodynamic Assay Development and Implementation Section
PBS	=	Phosphate-Buffered Saline
QC	=	Quality Control
RT	=	Room Temperature
SOP	=	Standard Operating Procedure

4.0 INTRODUCTION

The EMT Panel IFA was developed to evaluate the Epithelial or Mesenchymal Phenotype of tumor tissue sections. The assay uses monoclonal antibodies to EMT biomarkers including E-Cadherin, Vimentin and β-Catenin directly conjugated to various Alexa Fluor dyes as reporters for immunostaining.

5.0 ROLES AND RESPONSIBILITIES

Laboratory Director/Supervisor	The Laboratory Director/Supervisor directs laboratory operations, supervises technical personnel and reporting of findings, and is responsible for the proper performance of all laboratory procedures. The Laboratory Director/Supervisor oversees the personnel who follow the SOPs within the laboratory and is responsible for ensuring the personnel are certified and have sufficient experience to handle clinical samples.
Assay Operator	An Assay Operator may be a Laboratory Technician/ Technologist, Research Associate, or Laboratory Scientist who has been trained on this SOP. The Assay Operator works under the guidance of the Laboratory Director/Supervisor. This person performs laboratory procedures and examinations in accordance with the current SOP(s), as well as any other procedures conducted by a laboratory, including maintaining equipment and records and performing quality assurance activities related to performance.

5.1 It is the responsibility of the Laboratory Director/Supervisor to ensure that all personnel have documented training and qualification on this SOP prior to the actual handling and processing of samples from clinical trial patients. The Laboratory Director/Supervisor is responsible for ensuring the Certified Assay Operator running the SOP has sufficient experience to handle and analyze clinical samples.

5.2 The Assay Operator for this SOP should be well versed and comfortable with operation of the Bond-RX System.

5.3 Digital versions of the Bond-RX slide information and staining process should be printed, including the slide event log and the first page of the slide detail log. The printed logs must be attached to the Batch Record in order to maintain a complete audit trail.

5.4 The Certified Assay Operator responsible for conducting the assay is to follow this SOP and complete the required tasks and associated documentation. The Batch Record (Appendix 1) must be completed in ***real-time*** for each experimental run, with **each page** having the **patient ID** and **CTEP/Protocol ID**, as well as, ***dated and initialed.***

5.5 All responsible personnel are to check the DCTD Biomarkers Web site (https://dctd.cancer.gov/ResearchResources/ResearchResources-biomarkers.htm) to verify that the most recent version of the SOP for the assay is being used.

6.0 MATERIALS AND EQUIPMENT REQUIRED

6.1 PADIS /IQC-Supplied Critical Reagents and Calibrator/control slides

6.1.1 **Anti-E-Cadherin Alexa Fluor® 488 (IQC Part#: 30023)**: Anti- E-Cadherin, mouse Ab clone 36, conjugated to Alexa Fluor 488 (AF488), BD Biosciences, Cat#: 560061

6.1.2 **Anti-β-Catenin Alexa Fluor 546 (IQC Part#: 30025)**: Anti-β-Catenin (CTNNB1), rabbit Ab clone E247 (Abcam) custom conjugated to Alexa Fluor 546 (AF546) (Life Technologies)

6.1.3 **Anti-Vimentin Alexa Fluor 647 (IQC Part#: 30022)**: Anti-Vimentin, mouse Ab clone V9, conjugated to Alexa Fluor 647 (AF647) Santa Cruz Biotechnology, Cat # sc 6260 AF647S

6.1.4 **EMT Control slides (IQC Part#: 60007)**

6.2 DAPI dihydrochloride, FluoroPure™ grade (Invitrogen, Cat#: D21490)

6.3 Pipettes (100-1000 µL, 50-200 µL, 2-20 µL) and tips

6.4 50-mL polypropylene tubes (e.g., Becton Dickinson, Cat#: 352098)

6.5 Premium cover glasses, approx. 50 mm x 22 mm (e.g., Fisher Scientific, Cat#: 12-548-5E; Thermo Scientific; Cat#: 12440S)

6.6 Kimwipes (e.g., Fischer Scientific, Cat#: 06-666A)

6.7 Slide mailer/folder (e.g., Leica Microsystems, Cat#: 3802617)

6.8 Sterile-filtered, molecular biology grade deionized (DI) water (e.g., Invitrogen, Cat#: 10977-015)

6.9 10X phosphate-buffered saline (PBS; e.g., Invitrogen, Cat#: 70013-073) [Dilute 1:10 in DI water to prepare 1X PBS for use in assay.]

6.10 Anhydrous ethanol, histology grade [Fisher Scientific, Cat#: A405-20 (Filtered using 0.22 µm pore size before use)]; ACS/USP Grade can be purchased and used without filtration (Pharmco-AAPER, Cat#: 111000200PL05)

6.11 Xylene, ACS grade (e.g. EMD Millipore, Cat# XX0055-3)

6.12 ProLong® Gold antifade reagent (Invitrogen, Cat#: P36930)

6.13 Bond-RX™ Autostainer (Leica Microsystems, Cat#: 21.2701)

6.14 Bond Dewax Solution (Leica Microsystems, Cat#: AR9222)

6.15 Bond Epitope Retrieval Solution 1 (Leica Microsystems, Cat#: AR9961)

6.16 Bond Open Container – 10 pack; 30 mL (Leica Microsystems, Cat#: OP309700); alternate container sizes are listed in Batch Record (Appendix 1, Section 3)

6.17 Bond Research Detection Kit (Leica Microsystems, Cat#: DS9455)

6.18 Bond Primary Antibody Diluent (Leica Microsystems, Cat#: AR9352)

6.19 Normal Goat Serum Blocking Solution (e.g. Vector Laboratories, Cat#: S-1000)

6.20 Bond Universal Covertiles, 160 Pack (Leica Microsystems, Cat#: S21.4611)

6.21 Bond Wash Solution 10X Concentrate (Leica Microsystems, Cat#: AR9590)

6.22 Bond Universal Slide Labels and Printing Ribbon Kit (Leica Microsystems, Cat#: S21.4564.A)

6.23 Tissue-Tek® Slide Staining Dish White (Sakura, Cat#: 4457)

6.24 Tissue-Tek® 24-Slide Holder with Detachable Handle (Sakura, Cat#: 4465)

6.25 -80°C and -20°C freezers

6.26 2°C - 8°C refrigerator

6.27 Clinical slides prepared following SOP340550 with paraffin-embedded biopsy samples and control tissues on each slide

7.0 OPERATING PROCEDURES

7.1 Record the name of the assay operator, the facility running the SOP, and serial or NCI property tag number of Bond-RX™ in the Batch Record (Appendix 1).

7.2 If slides were shipped from a separate site, save the clinical shipping manifest for the laboratory record and attach a copy to the Batch Record.

7.3 Record the unique Patient ID(s) and CTEP/Protocol #(s) for the slides being assayed on each page of the Batch Record. This SOP and its associated Batch Record are sufficient for up to three Bond-RX™ slide trays containing a minimum of 1 Control slide per tray and up to 27 patient slides.

If more than one patients' slides were stained during a single run on the Bond -RX™, all patient ID(s) & protocol #(s) must appear on all pages of the Batch Record. If necessary, individual patient batch records can be generated by making copies of the original Batch Record.

7.4 Prior to beginning the assay, read the SOP and ensure sufficient materials and reagents are in stock to run the SOP. All reagents are to be prepared for use in one experimental run, and only in the amounts required for the specific assay.

7.5 Critical Reagents

7.5.1 Record the date of receipt, lot numbers, stock/supplied reagent concentration, recommended working concentration, recommended dilution and retest dates for the critical reagents in the Batch Record (Appendix 1, Section 1A).

7.5.2 All Critical Reagents are to be labeled with the date of receipt and stored under the specified conditions for no longer than the recommended duration.

- Storage conditions and retest dates for all Critical Reagents are provided on the shipping manifest that accompanies the critical reagent shipment.
- If the critical reagents are purchased directly from the manufacturer, Certified assay sites must qualify the reagents prior to use in the assay. Lot-to-lot differences, particularly for primary antibodies, are expected.

7.5.3 **Anti-E-Cadherin AF488:** Mouse monoclonal antibody (clone 36) conjugated to Alexa Fluor 488 is supplied by the manufacturer (BD Biosciences) as a stock solution in storage buffer (aqueous buffered solution containing BSA, protein stabilizer, and ≤0.09% sodium azide). Actual concentration as well as the recommended working concentration of the antibody will be provided by lot.

7.5.4 **Anti- β-Catenin-AF546:** Rabbit monoclonal antibody (clone E247) (Abcam) custom conjugated to Alexa Fluor 546 (Life Technologies) is supplied as a stock solution in storage buffer (0.1 M NaPi, 0.1 M NaCl, pH 7.5, 2 mM azide). Actual concentration as well as the recommended working concentration of the antibody will be provided by lot.

7.5.5 **Anti-Vimentin-AF647:** Mouse monoclonal antibody (clone V9) conjugated to Alexa Fluor 647 is supplied by the manufacturer (Santa Cruz) as a stock solution in storage buffer (PBS with < 0.1% sodium azide and 0.1% gelatin). Actual concentration as well as the recommended working concentration of the antibody will be provided by lot.

7.5.6 **Control slides** store in a desiccator at 2°C to 8°C away from volatile chemicals. Each clinical run should include at least one EMT control slide per slide tray.

7.6 If not already done, program the following information into the BOND-RX™ System prior to experimental setup:

7.6.1 Facility or laboratory running the assay should be added to the "Researchers List", which can be done from the **Add Study** screen in **step 7.10.1.2.**

7.6.2 To enter any new antibodies and Bond Open Containers, see Appendix 2, Section 2 for instructions.

7.6.3 Verify the EMT Panel IFA staining protocol is listed, and that it matches that listed in Appendix 2, Section 3A.

7.6.4 Verify that the HIER Protocol "HIER 10 min with ER 1" matches that listed in Appendix 2, Section 3C.

7.6.5 If a new Research Detection Kit is being used, scan the bar code to open the **Add Reagent** dialog box. Select the name of the reagent from the **Reagent name** drop-down list select "Wash Buffer" for the Open Container and in the expiration selection put a future date (suggest 1 yr. after date scanned).

7.7 Control and Clinical Slides

7.7.1 For clinical slide runs, one EMT control slide is recommended for each Bond-RX slide tray in use.

7.7.2 Clinical samples for this assay will be frozen needle biopsies collected according to SOP340507 and formalin-fixed, paraffin-embedded and sectioned according to SOP340550. A minimum of two clinical slides from each biopsy sample must be analyzed in order to report biomarker data, and normally three to four slides are recommended for staining and analysis for each patient slide set to ensure that an adequate cell count is achieved for each clinical biopsy. When possible, the slides are positioned in the slide trays so that slides from a single patient are contained within one Bond-RX slide tray.

7.8 Preparation of Reagents

7.8.1 During reagent preparation, be sure to note the lot number/serial number, retest date, and preparation date in the Batch Record (Appendix 1, Section 1B). Label all reagents with date of receipt and store under the specified conditions for no longer than the recommended durations.

Note: Some of the following reagents may be prepared ahead of time.

7.8.2 DAPI stock solution is provided as a 14.3 mM (5 mg/mL) solution in DI water. Aliquots can be stored at -20°C for up to 1 y; thawed aliquots can be stored at 4°C for up to 3 months. Light sensitive; protect all solutions from light.

7.8.3 1X Bond Wash Solution

7.8.3.1 Make 1 L of 1X solution by adding 100 mL Bond 10X Wash Solution to 900 mL DI water. Mix the solution until it is homogenous and label the bottle as "1X Bond Wash Solution" with the lot number and preparation date. Store Bond 1X and 10X Wash Solutions at 2 ºC to 8°C out of direct sunlight. 1X Bond Wash Solution can be used for 4 months.

7.8.3.2 When ready for use, 1X Bond Wash Solution can be poured into the bulk container marked "Wash Buffer" located within the Bond-RX™ Processing Module.

7.8.4 Research Detection Kit

7.8.4.1 Add 30 mL of 1X Bond Wash Solution to the 30-mL Open Container in the kit. Note: This container is required to be loaded with the Research Detection Kit, and only used for the first Bond wash.

7.8.5 Make sure that all required bulk reagent containers have sufficient volumes before starting the Bond-RX™ staining procedure. The bulk reagent containers should be at least a quarter full.

7.8.5.1 The bulk reagents include: 1X Bond Wash Solution, Bond Dewax solution anhydrous ethanol, DI water, and Bond Epitope Retrieval (ER) Solution 1. Note: all bulk reagents, including those not used (e.g. Bond ER Solution 2) need to be loaded into the Bond-RX with sufficient levels to perform the staining run.

7.8.5.2 When not in use, 1X Bond Wash Solution and ER Solution 1 containers are stored in a 2ºC to 8°C refrigerator, and the other bulk reagent containers are stored in the Bond-RX™ bulk reagent cavity.

Pre-warming the solutions stored in the refrigerator is not required; temperature does not adversely affect staining.

7.8.6 **Visually inspect all solutions for assay** to ensure there is no cloudiness or precipitate present. If they are cloudy or have precipitates, discard the solutions and clean the bottles with a mild bleach solution. Rinse the containers thoroughly with water before reuse.

7.9 Preparation of Antibody and Ancillary Working Solutions

7.9.1 Label two titration containers or open containers for the assay working solutions as follows: "**EMT Marker**" and "**Normal Goat Serum**". The container size is dependent upon the number of slides to be stained in a run; refer to Appendix 1, Section 3 for container volumes. The container labels correspond to the steps programmed into the staining protocol (Appendix 2, Section 2).

7.9.2 Record all working solutions and antibody information in the Batch Record (Appendix 1, Section 1).

7.9.3 Perform the calculations in the Batch Record (Appendix 1, Section 3) to prepare the antibody and the blocking agent working solutions as follows:

7.9.3.1 EMT Marker Working Solution

- **E-Cadherin-AF488**, **Beta-catenin-AF546** and **Vimentin-AF647** Ab Working Solutions should be prepared fresh for each run using Bond **Primary Antibody Diluent**. This will be used as "EMT Marker" in the staining protocol.
- The stock concentration and recommended working concentration for each of the antibodies will be provided by lot.
- To be sure there is sufficient volume for all of the slides to be stained, perform the calculations in the Batch Record (Appendix 1, Section 3)**.**
- Briefly warm the E-Cadherin-AF488, β-Catenin-AF546 and Vimentin-AF647 antibodies to room temperature and then pipette the calculated volume of Bond Primary Antibody Diluent and the three antibodies into the "EMT Marker" Container; record the preparation date in the Batch Record (Appendix 1, Section 3)**.**

7.9.3.2 Normal Goat Serum Block Working Solution

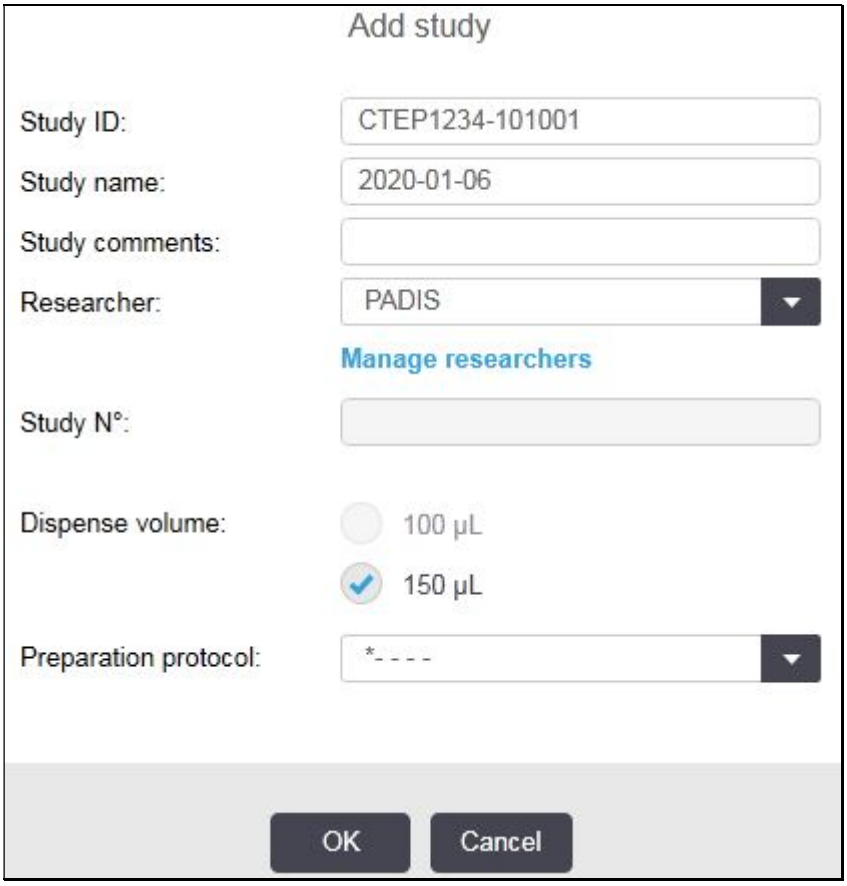
- The **Normal Goat Serum** Working Solution should be prepared fresh to a 10% final concentration in 1X Bond Wash Solution.
- Briefly warm the Goat Serum Block vial, and then pipette the calculated volumes of Goat Serum and 1X Bond Wash Solution into the "Normal Goat Serum" Open Container; record the preparation date in the Batch Record (Appendix 1, Section 3).

7.10 Protocol for Slide Staining in Bond-RX™ Processing Module and Offline Dewaxing

7.10.1 System Setup for Bond-RX™ Run

7.10.1.1 **Turn on** the computer and **open** the Bond software by clicking on the Bond icon, then **turn on** the Bond-RX™ Processing Module.

7.10.1.2 In the Bond software, select the **Slide Setup Screen,** and then select the **Add Study** button. In the **Add Study** window, change the fields as suggested in the table below and then click **OK**:

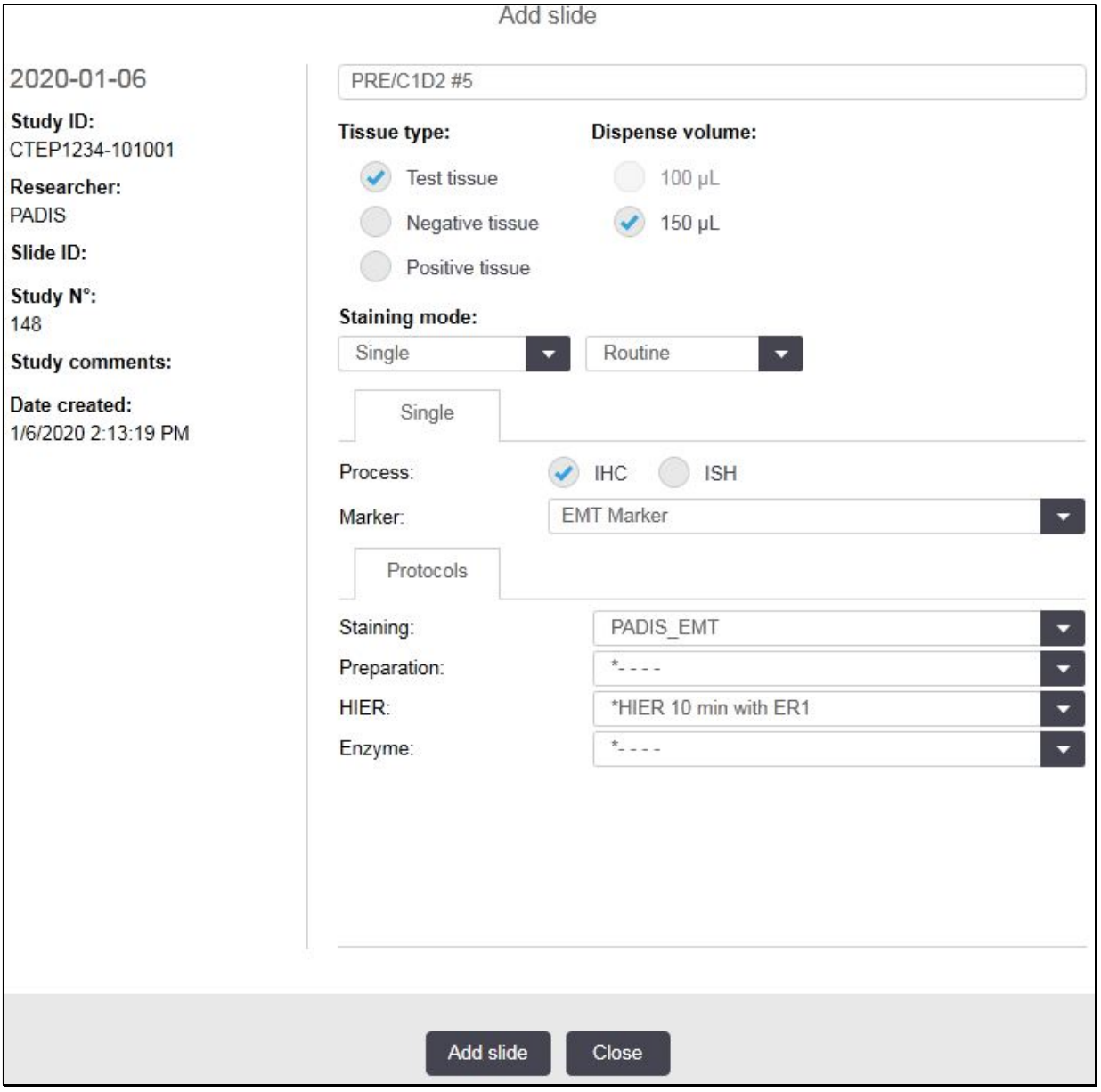


Field	Fill in
StudyID	CTEP#-Patient ID(s) (e.g., CTEP1234-101001)**
Study Name	Date of sample processing (e.g., 2020-01-06)
Study Comments	N/A
Researcher	Facility or laboratory running assay (from drop-down list)
Dispense Volume	150 µL
Preparation Protocol	Select " ----- " (if off-line Dewax) (If slides were not dipped and off-line dewaxed, use *Dewax)

** A new study will need to be added for each patient so that the slide label reflects the appropriate patient.

7.10.2 Add Slides to Bond-RX Run

7.10.2.1 While still in the **Slide Setup Screen**, click the **Add Slide** button, and in the **Add Slide** window, change the fields as shown in the table below:


Add slide
2020-01-06
Study ID:
CTEP1234-101001
Researcher:
PADIS
Slide ID:
Study N°:
148
Study comments:
Date created:
1/6/2020 2:13:19 PM
PRE/C1D2 #5
Tissue type:
Test tissue
Negative tissue
Positive tissue
Dispense volume:
100 µL
150 µL
Staining mode:
Single
Routine
Single
Process:
IHC
ISH
Marker:
EMT Marker
Protocols
Staining:
PADIS_EMT
Preparation:
*- - - -
HIER:
*HIER 10 min with ER1
Enzyme:
*- - - -
Add slide
Close

Field	Fill in / Select
Slide ID	**Automatically generated**
Study N*	**Automatically generated**
Study Name	**Date****
Study ID	**CTEP#(s) -Patient ID(s) (**)**
Comments	**Sample Time-point(s) & Slide # (e.g., Pre/C1D2 H24 #5) * THE SLIDE NUMBER MUST BE CHANGED FOR EACH SLIDE LABEL**
Tissue type	**Select: Test tissue (patient slides)** **Positive tissue (control slide)**
Dispense Volume	**150 µL***
Staining Mode	**Single Research**
Process	**IHC**
Marker	**Name of Marker(s) (e.g., EMT Marker)**
Staining	**Staining Protocol (e.g., EMT Marker Panel)**
Protocol	**Select " ----- "(if off-line Dewax) (If slides were not paraffin dipped and off-line dewaxed, use *Dewax)**
HIER	**Select Epitope Retrieval Method (e.g., HIER 10 min with ER1)**
Enzyme	**"*----"**

** *Automatically generated from Add Study screen*

7.10.2.2 For each new slide, a **Bond Slide ID Number** will be assigned automatically and is listed in the upper left-hand corner of the window.

7.10.2.3 For additional slides, click the **Add Slide** button at the bottom of the window. **Before adding slide change the slide #.**

7.10.2.4 Once all slides are entered, click **Close.**

7.10.2.5 Select the **Print Labels** button at the bottom of the screen to print the labels for the slides. Select **This Case** and click **OK.** If a label does not print correctly, right-click on the label and select **Print Label**.

7.10.3 Labels should appear with the following information:

Pre/D1H2 #5
CTEP8888- 34
EMT
00E7 07E
8/11/2016

7.10.3.1 Labels may need to be modified to get all the critical information on the label, and to get it in the correct order. To modify the label, click the **Bond Admin** icon, select **Labels,** and select the **PADIS** label template, as shown below. Click **Activate** to use this layout then exit the Bond Admin window.

7.10.3.2 Affix the printed Bond labels to the appropriate slides. Put the slides in the designated tray, and ensure they are aligned squarely with the inside edges of the slide tray so that the Processing Module can scan the information.

7.10.4 Offline Dewax of Paraffin Dipped Slides

7.10.4.1 This procedure should be followed for all tumor biopsy slides and control slides that have been dipped in paraffin to prolong stability.

- For slides that have not been dipped in paraffin, the dewaxing procedure should be carried out on the Bond-RX Automatic Staining System using the program detailed in Appendix 2, Section 3B.

7.10.4.2 Prepare the reagents for the off-line dewaxing procedure in Tissue-Tek Staining dishes and place the clinical and control slides in a Tissue-Tek slide rack. Deparaffinize, rehydrate and rinse slides as follows:

Number of Containers	Volume and Reagent	Incubation Time
4	200 mL Xylenes	10 min each
4	200 mL Anhydrous ethanol	3 min each
3	200 mL 95% Ethanol	3 min each
3	200 mL DI water	2 min each
1	1X Bond Wash Buffer	Final wash

7.10.4.3 Record the time of initiation of the dewaxing procedure, check off the appropriate box to acknowledge the completion of each incubation step, and record the time the slide rack is placed in the Bond Wash Buffer in the Batch Record (Appendix 1, Section 2).

7.10.5 Remove one slide from the Bond Wash Buffer and place it in the appropriate position on a Bond Slide Tray (minimum of 1 control slide is required for each clinical Bond-RX™ run and will normally be placed in the last position of each slide tray in use). Hold a covertile at about a 20° angle above the slide, placing the wicking end of the covertile on the bottom of the frosted end of the slide.

7.10.5.1 Using a transfer pipette gently apply 1X Bond Wash Buffer to the tip of the covertile and continue flush while carefully lowering the covertile onto the slide.

7.10.5.2 If bubbles are introduced, remove covertile and repeat application with a fresh covertile.

7.10.5.3 Obtain next slide from the Tissue-Tek container in Bond Wash Buffer, place onto the Bond Slide Tray and repeat above covertile application process.

7.10.5.4 Load Bond Slide Trays into the Bond-RX™ processing modules until the trays lock.

7.10.6 Add and Load Reagents for Bond-RX™ Run

7.10.6.1 Go back to the Bond main menu and select the **Reagent** icon. Using the hand-held scanner, scan the Research Detection Kit and antibody working solution containers to enter them into the Processing Module software inventory list.

- If you are using an Open Container or Research Detection Kit that is already in the Reagent list, after scanning the Container/vial the Bond-RX™ interface will report the remaining volume (inventory) in that container. If this is **sufficient volume** for your current run, proceed to the next step.
- Otherwise, click **Refill** in the pop-up window before placing the containers in the Processing Module. Note: 30-mL Open Containers can only be refilled to a 40-mL volume maximum.

7.10.6.2 Place the Open Containers containing “EMT Marker” and “Normal Goat Serum” working solutions into a reagent tray, then slide the reagent tray into a reagent tray slot at the front of the machine and lock into position. These containers can also be added to the tray containing the Research Detection Kit.

7.10.6.3 Place the Research Detection Kit with the Open Container containing “Wash Buffer” and working solutions into the reagent tray slot at the front of the Bond-RX™ and lock into position; the Wash Buffer Container needs to be placed into the first position of the reagent tray. The Processing Module will scan the reagent container bar codes to verify loading.

7.10.6.4 For each loaded tray in the processing module press the **Load/Unload** button on the front of Bond-RX™ below each tray slot to initiate scanning of the slide labels (Note *tray numbering – e.g. Tray 3 will load into the Processing Module closest to the reagent trays*.)

Note: Once slides are loaded into the Processing Module, the staining procedure needs to be started within 15 min or new slide labels will need to be assigned.

7.10.6.5 Once scanned, go to the computer screen and ensure that all labels were read correctly. If a slide label was not read correctly, right-click the corresponding slide and manually select the **Bond Slide ID** in the window.

7.10.7 Once all slides and reagent containers have been scanned, the **Play** button (triangle) will activate on the **System Status Screen** on the computer. Click the **Play** button on the screen to start processing the slides. **Note**: If the **Play** button does not light up, recheck that all trays are loaded correctly and that all containers have been scanned in. An error message will be displayed on the screen. Right-click on the error message and investigate as necessary.

Note: If the Bond Universal Covertiles are sticking to the slides during the staining procedure (they normally slide back and forth), it is likely that there is contamination in one of the bulk reagent solutions. Discard slides and all solutions. Clean bulk reagent bottles with a mild soap solution and then rinse thoroughly with water before reuse.

7.11 Completion of Bond-RX™ Staining Run

7.11.1 Allow the Prolong Gold Antifade Reagent to equilibrate to ambient temperature (*using a heat source to warm the vial is not recommended*). If the solution appears cloudy, discard according to your institution's safety guidelines and retrieve a fresh vial. Prolong Gold should be discarded 6 months after opening.

7.11.2 Just prior to slide staining completion, prepare two 250-mL Tissue-Tek staining dishes:

7.11.2.1 Fill the first staining dish with 200 mL DI water only. Place a Tissue-Tek 24-slide holder into this diH2O staining dish.

7.11.2.2 In second staining dish, prepare the DAPI Working Solution by adding 10 µL of the DAPI Stock Solution to 200 mL DI water, and mix thoroughly. Protect the solution from light by covering the entire dish with aluminum foil.

7.11.3 At the completion of the Bond-RX™ staining run, push the **Load/Unload** button to unlock the slide trays; remove the trays from the Processing Module. **Note:** Once the slides are removed from Processing Module, protect from light. Record the time the Slide Trays were removed from the Bond-RX™ Processing Module in Appendix 1, Section 4.

7.11.4 One slide at a time, remove the Bond Universal Covertile and immediately place the slide into the 24-slide holder immersed in the DI water staining dish.

7.11.5 Once all the slides are immersed in the DI water containing staining dish, transfer the rack to the DAPI Working Solution staining dish.

7.11.6 Incubate the slides for 50 min at ambient temperature in the dark (cover entire dish with aluminum foil) and gently agitate every 15 min. Record the DAPI staining start time in the Batch Record (Appendix 1, Section 4).

7.11.7 During the incubation time, fill three additional 250-mL staining dishes with 200 mL DI water each.

7.11.8 After the 50 min DAPI incubation step, remove the slide rack from the DAPI Working Solution and place it into a staining dish containing fresh DI water for 5 min. Record the time slides are removed from the DAPI Working Solution in the Batch Record (Appendix 1, Section 4).

7.11.8.1 Repeat the DI water wash process two additional times using a fresh DI water staining dish. Confirm the completion of wash steps in the Batch Record (Appendix 1, Section 4).

7.11.9 One slide at a time:

7.11.9.1 Transfer the slides to a paper towel and use a Kimwipe to wick away any residual liquid, taking care not to touch the tissue or let it dry out.

7.11.9.2 Using a 1000-µL pipette, place no more than two drops of Prolong Gold Antifade Reagent onto the sections and cover with a cover slip.

7.11.10 Place the slides in a slide book, lying flat in a safe location. Allow the slides to cure overnight in the dark at ambient temperature.

7.12 Slides should be stored in the dark at 2ºC to 8°C and imaged **within 72 h** after cover slipping.

7.13 Review and finalize the Batch Record and document **ANY** and **ALL** deviations from this SOP during the slide staining process in the Batch Record (Appendix 1, Section 5).

7.14 The Laboratory Director/Supervisor should review the Batch Record and sample reports and sign the Batch Record affirming the data contained within the reports are correct (Appendix 1, Section 7).

7.15 Clean-up

7.15.1 If this is the last experimental run of the day, be sure to **turn off** the Bond-RX™ Processing Module; this will ensure the lines are cleaned at the beginning of each new day when the module is turned back on. Empty the waste containers as needed.

7.15.2 Store ER Solution 1 and 1X Wash Solution bulk reagent bottles at 2ºC to 8°C. The rest of the bulk reagent containers can remain inside the body of the Bond-RX™ Processing Module.

7.15.3 Bond Open Containers can be rinsed and used for 40 mL total for the **same** reagent. It is recommended to always use fresh working solutions but working antibody solutions can be stored at 2ºC to 8°C and used for up to 5 d after preparation.

7.15.4 Place the Bond Universal Covertiles into anhydrous ethanol for 10 min and dry with a Kimwipe for reuse. If cracked or damaged, discard.

7.15.5 Make sure all Bond-RX™ daily maintenance procedures have been completed. For overall maintenance, clean the bulk reagent bottles with a mild bleach solution every 3-6 months; rinse thoroughly with water before reuse. Additionally, at least once per month perform Cleaning and Maintenance as outlined in the Leica Bond-RX™ User Manual.

8.0 OPTIONAL: SHIP TO CERTIFIED ASSAY SITE FOR ANALYSIS

If the IFA analysis will be performed at a separate certified assay site, ship the slides as follows:

IMPORTANT: Include a copy of the Batch Record for all samples being shipped with the Shipping Manifest.

8.1 Send an e-mail to the certified assay site prior to shipping to advise recipient of scheduled shipping time. Be sure to request and receive a confirmation e-mail prior to shipping.

8.2 Generate a shipping list containing all the specimen records using the Shipping Manifest template as shown in Appendix 3.

8.3 **Verify** that the contents of the package match the Shipping Manifest.

8.4 Print and attach the shipping address onto the outside of the shipping container.

8.5 Record the shipping date, time and tracking number in the Batch Record (Appendix 1, Section 6).

8.6 Ship the specimens **with a copy of** the Shipping Manifest and copies of the completed Batch Records for all patient specimens. Retain copies of the completed Shipping Manifest and Batch Records in your records.

APPENDIX 1: BATCH RECORD EMT PANEL IFA SLIDE STAINING

NOTE: *Record times using* ***military time*** *(24-h designation); for example, specify 16:15 to indicate 4:15 PM*

Certified Assay Operator: _____

Facility/Laboratory Running Assay: _____

Serial or NCI Property Tag Number of Bond RX: _____

Patient ID	CTEP/ Protocol #	Slide #

1. Reagents

A. Critical Reagents

Reagent Name	Date Received	Lot Number	Stock Reagent Conc.	Recommended Working Conc.	Recommended Dilution	Retest Date
E-Cadherin-AF488 Ab	/ /					/ /
β-catenin-AF546 Ab	/ /					/ /
Vimentin-AF647 Ab	/ /					/ /
Control Slides	/ /			N/A	N/A	/ /

BATCH RECORD: INITIALS _____ DATE: _____

Patient ID(s):
CTEP/Protocol ID(s):

B. Reagent Log

	Stock Solution		***Working Solution***	
Reagent	Lot#	Expiration Date	Concentration	Preparation Date
10X Bond Wash Solution		/ /	1X Solution	/ /
Bond Primary Antibody Diluent		/ /	N/A	N/A
DAPI		/ /	0.25 µg/mL	/ /

2. Off-line Dewaxing of Paraffin Dipped Slides

Were slides dipped in paraffin to prolong stability? ☐ Yes ☐ No

A. Off-line Dewax Reagent Applications

Record the times and acknowledge the reagent applications step below:

Step	**Time**
Time that Off-line Dewax Procedure Began	:
Four, 10 min Xylene Incubations Completed	☐
Four, 3 min Anhydrous Ethanol Incubations Completed	☐
Three, 3 min 95% Ethanol Incubations Completed	☐
Three, 2 min DI H_2O Rinses Completed	☐
Time Slides Placed in 1X Bond Wash Solution	:

Patient ID(s):
CTEP/Protocol ID(s):

3. Preparation of Working Solutions

Reagent Containers	**Max. Vol. (mL)**	**Minimum Required Residual Vol. (uL)**
Bond Open Containers, 30 mL (Leica Microsystems, Cat#: OP309700)	30	1500
Bond Titration Kit (Containers and Inserts; Leica Microsystems, Cat#: OPT9049)	6	300
Bond Open Containers, 7 mL (Leica Microsystems, Cat#: OP79193)	7	1000

Date (Prepared)	**Reagent Name**	**Diluent Name**	**A. Suggested Dilution**	**B**. Total # of ☐ slides X 300 µl	**C. Residual vol** (ul) 300µL, 1000µL, or 1500µL (see minimum requirement in the table above and enter below)	**D. Total Volume:** *(needed for staining)* **B+C** (µl)	**E. Vol. of reagent:** *total volume/dilution factor* **D ÷ A** (µl)	**F. Vol. of Diluent D – E*** (µl)
	1. E-Cadherin-AF488 Ab (EMT)	**Bond Primary Ab Diluent**						***(EMT Ab)** ***D–(E1+E2+E3)***
	2. Betacatenin-AF546 (EMT)							
	3. Vimentin-AF647 (EMT)							
Date (Prepared)	**Ancillary Reagent Name**	**Diluent Name**	**A. Suggested Dilution**	**B**. Total # ☐ slides X 150 µL	**C. Residual vol** (µL) (see above)	**D. Total Volume:** *(needed for staining)* **B+C** (µL)	**E. Vol. reagent:** *total volume/ dilution factor* **D ÷ A** (µL)	**F. Vol. of Diluent D – E** (µL)
	Goat Serum Block	1X Bond Wash	**1:10**					

After Bond-RX™ staining run is complete, print the Run Event log and the first page of the Run Detail Log*. Attach the documents to the batch records. ***if there was an adverse event during the** Bond-RX™ staining run the entire Run Detail Log should be printed and attached to the batch records*

BATCH RECORD: INITIALS _____ DATE: _____

Patient ID:
CTEP/Protocol ID:

4. Staining of Slides

A. DAPI Staining and Cover Slip Application

Just prior to staining with DAPI, prepare DAPI Working Solution by diluting 10 µL DAPI stock (5 mg/mL) into 200 mL DI water in a 250-mL staining dish. Discard excess Working Solution at end of the assay run.

	Time
Slide Trays Removed from Processing Module	:
DAPI Working Solution Added to Slides	:
DAPI Working Solution Removed	:
Three, 5 min DI Water Washes Completed	☐
ProLong Gold Antifade Reagent with Cover Slips Added	:

5. Notes, including any deviations from the SOP:

6. Shipping to Certified Assay Site

Date and time samples shipped: _____

Tracking information: _____

Attach copy of Shipping Manifest

7. Laboratory Director/Supervisor Review of Batch Record

Laboratory Director/Supervisor: _____ (PRINT)

_____ (SIGN)

Date: _____

APPENDIX 2: BOND-RX™ PROCESSING MODULE

1. Modifications to SOP for running a single slide tray in Bond-System

When using a Bond Titration Container with Insert, be sure to scan the bar code on the titration container when programming the Bond-RX System, and clearly label each container as Marker. The Bond Container Insert should be discarded after use, but the Bond Titration Container can be reused multiple times.

2. Register new antibodies and Bond Open Containers in the Bond-RX™ System

A. On the Reagent Screen, add "EMT Marker" and "Normal Goat Serum" to the reagent list as follows:

Field	EMT Panel	Goat Serum Block
Name:	*EMT Marker*	Normal Goat Serum
Abbreviated name:	*EMT*	*NGS*
Type:	*Primary*	*Ancillary*
Single/double stain	*Double*	*Single*
Default Staining protocol:	"EMT Panel"	*N/A*
Default HIER protocol:	*HIER 10 min with ER1*	*N/A*
Default enzyme protocol:	*- - -	*N/A*
Preferred	*Selected*	*Selected*

B. Scan the new Open Container, Titration Kit Container, or Research Detection Kit Container bar codes to open the **Add Reagent** dialog box. Select the appropriate reagent name from the **Reagent name** drop-down list and label the Containers with the antibody names for easy identification. Repeat this procedure for each of the assay reagents. The Containers will not need to be entered again until a new Container, and therefore a new barcode, is used.

3. Staining Protocols

Create the following staining protocol (A), "EMT Marker Panel", on the BOND-RX™ Processing Module. Protocols B and C are pre-programmed protocols on the BOND-RX™ Processing Module and will be used for the EMT Panel IFA.

A. Staining Protocol: "EMT Marker Panel" (protocol entered by user)

Solution	Temperature ºC	Time (min)*
Wash Buffer†	Ambient	0
Bond Wash Solution	Ambient	5
Bond Wash Solution	Ambient	0
Bond Wash Solution	Ambient	0
Normal Goat Serum	Ambient	20
EMT Marker	Ambient	30
EMT Marker	Ambient	30
Bond Wash Solution	Ambient	0
Bond Wash Solution	Ambient	5
Bond Wash Solution	Ambient	0
Bond Wash Solution	Ambient	0

**A time of zero indicates that the solution is applied, but that minimal time elapses before the next application.*

† The Bond-RX™ Processing Module requires one established solution to be used from its reagent selection list. For the Research Detection Kit, 1X Bond Wash Solution is placed into a 30-mL Open Container and is used in this protocol.

B. Preparation Protocol: "*Dewax" (using Processing Module preset protocol)

Solution	Temperature ºC	Time
Bond Dewax Solution	72	30 sec
Bond Dewax Solution	72	0
Bond Dewax Solution	Ambient	0
100% Ethanol	Ambient	0
100% Ethanol	Ambient	0
100% Ethanol	Ambient	0
Bond Wash Solution	Ambient	0
Bond Wash Solution	Ambient	0
Bond Wash Solution	Ambient	5 min

C. HIER Protocol: "*HIER 10 min with ER 1" (using Processing Module preset protocol)

Solution	Temperature ºC	Time (min)
Bond ER 1 Solution	Ambient	0
Bond ER 1 Solution	Ambient	0
Bond ER 1 Solution	100	10
Bond ER 1 Solution	(Cool-down phase)	12
Bond Wash Solution	35	0
Bond Wash Solution	35	0
Bond Wash Solution	35	0
Bond Wash Solution	Ambient	3

APPENDIX 3: EMT PANEL IFA STAIN SLIDES SHIPPING MANIFEST

Ship From: Contact Name: Tel: E-mail:	**Shipping Manifest**	**Ship To:** Attn: Tel: E-mail:

Shipping Date: **Carrier:**

In Package	Item No.	Patient ID	Clinical Protocol/CTEP#	Bond Slide ID	Comment
☒	*Example*	*Control Lot # or Patient 54*	*CTEP 8888*	*78AD*	*Control slide or Patient slide (Pre & D1H2)*
☐	1				
☐	2				
☐	3				
☐	4				
☐	5				
☐	6				
☐	7				
☐	8				
☐	9				
☐	10				