1.0 Purpose and Scope

1.1 This procedure will be a standard operating protocol for the use of the Abbott CEP Y Spectrum Orange DNA Probe to label male cells and recover the cells using the DEPArray NxT or PLUS

1.2 This procedure applies to the Bioforensics Laboratory located in Lyman Hall rooms 422 and 424.

2.0 Roles and Responsibilities

Role	Responsibility
Responsible Official	It is the duty of the Responsible Official (RO)/ Alternate Responsible Official (ARO) to ensure that this policy and procedure contain herein is read and understood by the appropriate laboratory personnel. The RO or ARO retains the ability to approve *Significant Protocol Deviations* when necessary. The RO/ARO must record the deviation in the ***Protocol Deviation log"*** It is the responsibility of the RO to ensure Bioforensics Laboratory Personnel complete the appropriate safety and bench training is obtained and completed in a manner that demonstrates competency. It is the responsibility of the RO and ARO to ensure the document herein is reviewed periodically to ensure continued relevance to the work being performed in the Bioforensics Laboratory.
Laboratory Personnel	It is the responsibility of the Bioforensics Laboratory Personnel to adhere to this policy and all other Bioforensics Laboratory and Syracuse University Policies. It is the responsibility of the Laboratory Personnel to obtain permission from the RO/ARO before implementing a *Significant Protocol Deviations*.

3.0 Definitions/Acronyms

3.1 *FISH* – *f*luorescent in-situ hybridization

3.2 *RT* – room temperature

3.3 *DI* – deionized

4.0 Safety Considerations

4.1 None

5.0 Procedure-Test Method

5.1 Standards/Controls and Reagent Preparation

5.1.1 20X SSC Buffer Preparation

a. Combine:

i. 66 g 20X SSC

ii. 200 mL sterile DI water

b. Adjust the pH to 5.3 with HCl

c. Filter through 0.45 µm filtration unit

d. Store at RT (up to 6 months)

5.1.2 0.4X SSC Wash Solution

a. Combine:

i. 20 mL 20X SSC pH 5.3

ii. 950 mL purified water

b. Adjust the pH to 7.0 to 7.5 with 1N NaOH

c. Filter through 0.45 µm filtration unit

d. Store at RT (up to 6 months)

5.1.3 0.1% Triton x-100 (10% w/v) in 20X SSC

a. Combine:

i. 1 mL 20X SSC pH 5.3

ii. 8.9 mL purified water

iii. 0.1 mL Triton x-100

b. Adjust the pH to 7.0 to 7.5 with 1N NaOH

c. Filter through 0.45 µm filtration unit

d. Store at RT (up to 6 months)

5.1.4 PBS with BSA

a. 1× phosphate buffered saline (PBS), 1% bovine serum albumin (BSA).

b. Weigh 0.05 g BSA and add 4 mL ice cold 1× PBS.

c. Mix until BSA has dissolved.

d. Make up to 5 mL with ice cold 1× PBS. Store at 4 ºC

5.1.5 1% BSA

a. Combine:

i. 0.05 g solid BSA

ii. 5 mL sterile dI water

5.2 Pre-Procedure Steps

5.2.1 DAPI dilution

a. Dilute the DAPI (1mg/mL) counterstain 1:10,000 if not yet diluted.

5.2.2 Set water bath to 73˚C.

5.2.3 Ensure the 1x PBS / 1% BSA solution used in step 5.5 is in the freezer at least one hour prior to the procedure.

5.3 Cell Suspension

5.3.1 Add 500µL of PBS into a lo-bind microcentrifuge tube and add the swab by cutting off the fabric portion into the tube.

5.3.2 Place tube in thermomixer and incubate at room temperature at 300rpm for 20 minutes.

5.3.3 Spin at 600g for 3 minutes to pellet the cells, carefully remove the supernatant.

5.3.4 Take note of the size of the pellet.

5.4 Fixation

5.4.1 Make 2% (formaldehyde) using 1% PBS.

5.4.2 Add 100µL of fixative to each tube slowly, pipet up and down for 15 seconds, and then add an additional 100µL and pipet up and down for 15 seconds again.

5.4.3 Incubate for 10 minutes at room temperature.

5.5 Cell Permeabilization

5.5.1 Add 500 µL ice cold 1x PBS / 1% BSA.

5.5.2 Spin at 600g for 3 minutes and remove and discard supernatant.

5.5.3 Resuspend cells in 500 µL ice cold 1x PBS / 1% BSA.

5.5.4 Spin at 600g for 3 minutes and remove and discard supernatant.

5.5.5 Resuspend cells in 200 µL 0.1% Triton x-100.

5.5.6 Spin at 600g for 3 minutes.

5.5.7 Remove and discard supernatant.

5.6 Hybridization of DNA to probe

5.6.1 For a 0.35x concentration (final volume = 40 µL):

a. Add 38.65 µL hybridization buffer to each tube.

b. Transfer the sample(s) into a 0.2mL PCR tube(s).

c. Add 1.35 µL CEP Y probe to each tube.

NOTE: all tubes that have the CEP Y probe should be kept out of light for the remainder of the protocol

d. Place samples in a 73˚C water bath for 5 minutes

e. Rotate samples on a laboratory tube rotator (rotisserie) at room temperature overnight.

   i. **IMPORTANT**: Ensure that the tubes are fully covered in foil and/or kept out of direct or indirect light.

f. Warm 0.4x Wash SSC Buffer at 73˚C for 10 minutes.

g. Transfer samples into lo-bind microcentrifuge tubes.

h. Wash the PCR tubes with 200 µL warmed 0.4x Wash SSC Buffer and add to the samples.

i. Incubate cells at 73˚C for 2 minutes to facilitate removal of excess probe that may have specifically bound to sequences partially homologous to target sequence.

j. Add 200 µL ice cold 1% BSA to cells to quickly drop the temperature and prevent clumping of cells.

k. Centrifuge at 600g for 3 minutes.

l. Remove and discard supernatant.

5.7 DAPI counterstain and slide application

5.7.1 Resuspend the cells in 100 µL ice cold 1x PBS / 1% BSA.

5.7.2 Spin at 600g for 3 minutes.

5.7.3 Remove and discard supernatant.

5.7.4 DAPI staining:

a. Add 25 µL of a 1:10,000 dilution of DAPI and incubate for 40 minutes at RT

b. Centrifuge at 600g for 3 minutes.

c. Remove and discard supernatant.

5.7.5 Post - DAPI counterstain

a. Add 100 µL 1% cold BSA to each sample.

b. Centrifuge at 600g for 3 minutes.

c. Remove and discard supernatant.

d. Add ~ 30 µL if ice cold 1% BSA to each sample.

e. (**Optional**) Apply ~ 5 µL of the sample to microscope slide and let air dry in the dark. The microscope slide should be used to check staining efficiency. The rest of the sample should be kept in suspension.

f. Add 3 µL Vectashield mounting media to microscope slide and coverslip.

NOTE: The sample can either be stored covered in foil and out of light at -20° C or it can be run on the DEPArray.

NOTE: If the sample will be run on the DEPArray the following day ensure that the room humidity is at least 50%. If it is not run humidifiers the day prior.

5.8 Stain Quality Check with Fluorescent Microscope (optional)

This procedure is to check the quality of the staining. It is critical that the DAPI staining is visible and stained a majority of the cells present. The number of cells stained with the Y probe will vary because it is dependent on the number of male cells in the sample. The internal protocol for the fluorescent microscope available at the laboratory should be used. See table 1 for recommended filters.

Table 1: Probe emission and excitation spectra with recommended filters.

Fluorescent Probe	Excitation Peak	Recommended Channels	Excitation wavelength (nm)	Emission wavelength (nm)
Spectrum Green™	497	FITC	469	510
Spectrum Orange™	559	PE/Cy3	546	581
DAPI counterstain	367	DAPI	376	447

5.9 DEPArray Protocol

Prior to preparing the sample for loading onto the DEPArray turn the sign into the DEPArray instrument account (on the DEPArray onboard computer) and select start 'Start. Instrument tests will be automatically performed. If they do not pass access the Menarini portal and complete a ticket. Note, low humidity (under 50%) is a common error experienced during the winter months.

5.9.1 Sample Preparation - *DEPArray Buffer Washes*

The DEPArray buffer washes are to remove the buffers/reagents that were used to stain the sample and to impart a negative charge on the cells to permit recovery on the DEPArray instrument).

a. Remove the DEPArray Buffer from freezer and thaw to room temperature
b. Place the DEPArray Buffer into a liquid bath sonicator and degas for 10 minutes (600s) - (see '*TRA_TUX_130-R1-DEPArray buffer for fixed cells degas procedure for use'* for further details).
c. Add 500 µL of DEPArray Buffer to the sample and centrifuge in a centrifuge. with a rotating bucket rotor for 3 minutes at 600g.
d. Once complete, remove supernatant leaving ~50 µL of buffer.
e. Repeat steps c. and d. 2 additional times for a total of 3 washes.
f. (**Optional**) Count the cells using a hemacytometer.
    i. The maximum number of cells loaded in the DEPArray forensic mode is 6000, with an optimal number between 2,000 to 3,000.

5.9.2 Sample Preparation - *Cartridge Loading*

The DEPArray Buffer and sample should be added to the cartridge using only Menarini approved micropipettes and tips to ensure proper fit into the cartridge ports. Follow the DEPArray Cartridge loading procedure (summarized below).

a. Open a new cartridge, making sure to save the outer packaging.
b. Load 2.5 mL of the DEPArray Buffer into port "B" slowly, make sure no air bubbles are introduced to the cartridge lines.
c. Load 12 µL of the sample into port "S" slowly, making sure no air bubbles are introduced into the cartridge lines.

5.9.3 DEPArray System Setup and Cartridge Loading

a. If the instrument is not powered on, turn the system on and sign into user account and select 'Start'.
b. Instrument tests will be automatically performed. If they do not pass access the Menarini portal and complete a ticket.
c. The instrument door will then open, and the cartridge can be placed into the drawer.
d. Wait for the prompt and scan the barcode of the gray cartridge packaging.
e. Skip the buffer labeling step however name the run (e.g., date_initials).
f. Select mode 'Forensics RUO' set to 'fixed' and 'enable'
g. Right click on 'Chip Scan' and add PE, DAPI and Brightfield to be the selected channels.
h. Right click on PE and select 'take picture PE' and set PE to faint signal
i. Right click on DAPI and select 'take picture DAPI' and ensure DAPI set to bright signal.
j. Right click on Brightfield and select 'take picture Brightfield'.
k. Add a second PE & DAPI channel (PE1 and DAPI1) and select 'take picture' for each.
l. Begin Sample Load
    i. Wait until Sample load reaches 100% to proceed to the next step. Note, it typically pauses at 7% and ~70%.

5.9.4 Sample Scan

a. Monitor the sample scan until several fields with cells are scanned.

b. Pause the sample scan and select cages in the bottom right of the main chamber (most cells are located around the outer corners of the chamber).

   i. Set parameters (exposure, camera gain, lamp intensity, offset) to best provide a visible signal in both PE (PE1) and DAPI (DAPI1) channels.

   ii. Set an offset at a different µm for the PE1 and DAPI1 channels so that there are 2 focal points for the cells (e.g., PE and DAPI can be set at 55 µm and PE1 and DAPI1 can be set at 35 µm).

c. Resume Sample Scan until complete (~30-40 minutes).

5.9.5 Cell Selection

a. Select 'parameters' on the top left and select signal and max intensities for PE and DAPI channels to set up a table with all cells on the left side of the screen.

b. Select the channels on the bottom of the screen (APC, PE, PE1, DAPI, DAPI1, Brightfield, an overlay of PE and DAPI and an overlay of PE1 and DAPI1),

   i. Set the colors to your preference (PE = orange, DAPI = blue).

c. To create new tables, select 'New Table' at the top of the panel.

   i. For example: Table 0 – for 'male only cells' – single cells with signal in PE channel; Table 1 – for 'mixed clumps' – multiple cells with at least one signal in the PE channel; Table 2 – 'unknown' cells that could have signal in PE channel, but not distinct; Table 3 – 'female only' – single cells with no signal in PE channel.

d. Sort the Cell Panel by PE intensity (descending) by selecting the PE column header.

e. Manually review the cells and use a Hot Key to send the selected cell to its intended table.

   i. The Hot Key is the number next to its associated table in brackets (e.g.,

Table [0] has a Hot Key of 0. Group the cell with its intended table.

ii. To deselect a cell from the table, press the associated Hot Key again.

f. **(Optional) Cell Panel – Saving images**

i. Once the cells are in their respective groups, navigate to the Cell Panel and Select a table to view the cells in the selected table.

ii. To save, select the camera icon on the left, choose the .tiff extension, choose each channel individually, then select the icon on the bottom left to save it to the run file, then select OK.

iii. Repeat for all channels that are to be saved (PE, DAPI, PE1, DAPI1, Brightfield, overlay of PE/DAPI and overlay of PE1 / DAPI1).

5.9.6 Routing

Note : 'Group 1' is generically used as a label for the groups of cells recovered in the each of the tables set in previous steps.

a. Select the '+' button next to the table to view the selected cells with their Cell ID.

b. On the bottom left, open the shutter and look at the live view of the chamber.

c. Set the parking speed to 1000ms (this slows down the speed of the routing but minimizes the chance the cells are lost during the routing process).

d. Select on obstacles to see any possible obstacles that surround the cells.

i. Select on Group 1 and select move to parking and press the start button to begin the parking process.

ii. Observe the cells in the live configuration and pause the routing if any cell is lost from its Cell ID during this stage.

e. Repeat for all tables

i. Park each group individually.

ii. To do this, individual cells can be selected and then moved to parking.

iii. Take a picture of the live configuration once the cells are parked to see if there was any cell loss.

5.9.7 Recovery

a. On the top left, set up recovery configuration and select 200 µL recovery tubes.

b. Navigate to the option to open the recovery support (On the bottom left of the pop-up icon).

   i. To do this, select the Trays on the DEPArray screen, then open recovery

   ii. Load the proper amount of 200 µL PCR recovery tubes with an extra in A1 for the primer (the primer is to start to reagent drop to ensure proper elution of the cells). Tubes should be placed in every other column and every other row.

   iii. For example: 5 tubes total → A1 – primer; A3 – Group 1; A5 – Group 2; A7 – Group 3; A9 – Group 4.

c. The number of drops is automatic but ensure the proper number of drops under the recovery schematic are selected (see DEPArray User Guide for more details).

d. To begin recovery, right click on A1 and set the primer.

   i. This will wash the main chamber of the remaining cells and set the primer in A1.

e. To recover the Groups, select Group 1 and drag it to A3.

f. Repeat for the other Groups.

   i. Note, if the group is split and identified with different colored cells, they can be dragged individually based on the color to separate recovery tubes.

g. Once the recovery support complete, right click on Group 1 in the center of the screen and move to recovery.

   i. Repeat for the rest of the groups.

h. Once recovered, remove the support tubes.

i. Stop the run and the cartridge can be discarded and system can be shut down.

j. Proceed to volume reduction (See Menarini Volume Reduction Manual).

6.0 Referenced Documents

Policies, Procedures, and Work Instructions	Templates & Forms	Examples
DEPArray NxT User Manual	Version 3.0	
W_MKT_051 DEPArray™NxT Volume Reduction Protocol for Fixed Cells Instructions for Users	Version 1.1	
TRA_TUX_130-R1-DEPArray buffer for fixed cells degas procedure_for use	Rev. 1	

7.0 Records

Record	Responsibility	Location	Retention	Disposition
NA	NA	NA	NA	NA

8.0 Standards Mapping

Standard	Requirements
NA	**NA**

9.0 Revision History

Date	Item	Description
NA	NA	NA