Document Number: **SASoM/EQUIP/026.v2**

Title: **Image Analysis using HistoRx® PM-2000, AQUA (Automated Quantitative Analysis)**

Version: **v2**

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Effective from:	01/01/2018
Valid to:	31/12/2022

SOP History		
Number	Date	Reason for Change
v1	01/01/2013	Original
V2	01/01/2018	Update

1.0 Purpose –
The purpose of this SOP is to outline the principles of image analysis using HistoRx ® PM-2000, AQUA in Laboratory 248 at the St Andrews School of Medicine (SASoM).

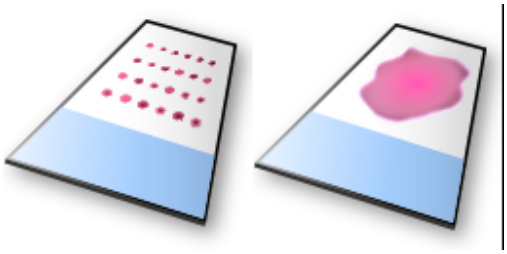
2.0 Scope –
This SOP applies to routine image analysis using HistoRx ® PM-2000, AQUA in the SASoM.

3.0 Responsibilities –
It is the responsibility of all users of the AQUA within the SASoM to comply with this SOP.

4.0 Procedure –
AQUAnalysis:
Double-click the **AQUAnalysis** icon on the desktop to start the program.

On the **File** menu, click **Load TMA**.

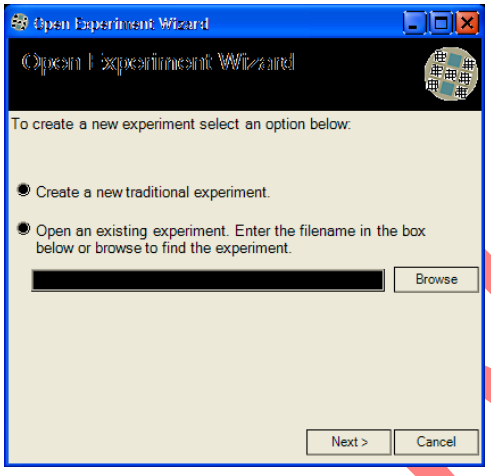
Browse for the TMA file created in the previous section, and then click **OK**.



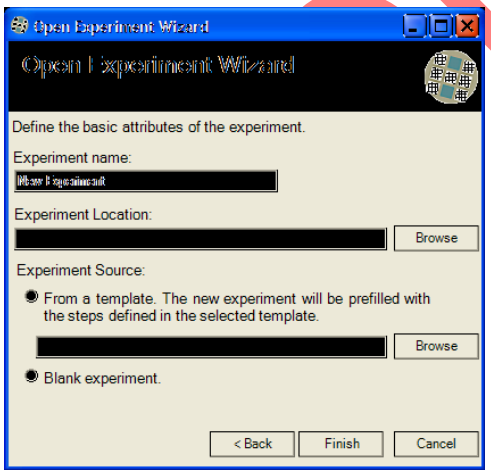
The selected TMA file appears in the **Slide Information** pane on the *AQUAnalysis* **Main** window.

NOTE: Multiple TMA files can be loaded simultaneously into the AQUAnalysis program if they contain images from an identical set of filters. Only one TMA file is active at any one time. To activate a different loaded TMA file, click on the corresponding slide icon. The eye icon marks the active TMA file.

<u>Creating a New Experiment:</u>



On the **File** menu, click **Open Experiment Wizard**.



Select **Create a new traditional experiment**, then click **Next**.

In the **Experiment name** text box type in a name for the experiment.

Click the **Browse** button to the right of the **Experiment Location** text box then select a directory for experiment data to be stored.

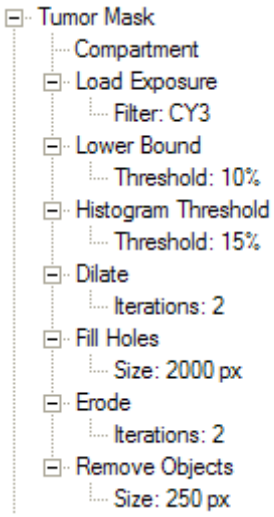
Select From template then open the browse to find previously set experimental template located in Experimental template in AQUA folder in Y: drive as an experiment source. Then click **Finish** to complete the wizard.

TIP: Frequently used experimental setups can be saved as AQUA templates files and then loaded in this step.

Experiment Steps and Procedures:

The combination of steps and procedures detailed below is a standard experimental setup for epithelial tissue analysis and is contained in the *Standard HARP AQUA template* file. Values in the following AQUA experimental setup steps are recommendations. The user should define variable values for procedures such as **Histogram Threshold**, Fill **Holes**, **Remove Objects**, etc., to reflect the staining and tissue properties of the TMA file being analyzed.

a. Tumour mask



Load Cy3 exposure then run Lower bound, Histogram threshold, dilate, fill holes, erode, remove objects step by step.

The **Lower Bound** procedure removes the lowest *X* percentage of the pixel range recorded in the image, effectively setting them to zero. This can be useful for removing background fluorescence haze. The recommended value for the **Threshold** variable is 10%.

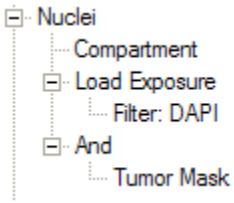
The **Histogram Threshold** procedure sets all pixels with CY3 signal above the chosen threshold to ON\1\white and all other pixels to OFF\0\black, effectively binerizing the image. The recommended value for the **Threshold** variable is 15%.

The **Dilate** procedure blooms all non-negative pixel areas by the number of pixels specified in the **Iterations** variable. The recommended value for the **Iterations** variable is 2.
The **Fill Holes** procedure fills the nuclei holes in the cytokeratin stain. The recommended value for the **Size** variable is 2000px.

The **Erode** procedure contracts all pixels bloomed by the **Dilate** procedure in Step 16 above, with the exception of those pixels now part of a filled hole. The recommended value for the **Iterations** variable is 2.

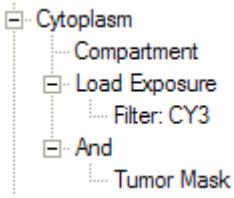
The **Remove Objects** procedure cleans up the mask by removing small, stand-alone clusters of positive pixels. The recommended value for the **Size** variable is `250` px.

b. Nuclei



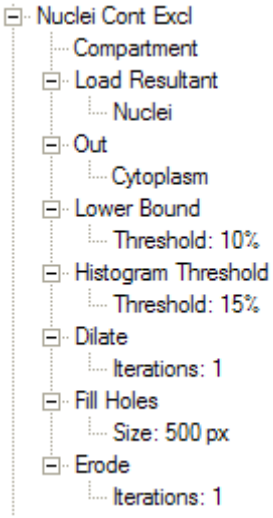
After loading Dapi exposure run And tumour mask.
The **And** procedure output is a multiplication of the matching pixels from the exposure loaded at the beginning of the step and those of the variable resultant image.

c. Cytoplasm



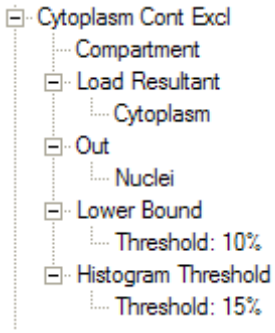
d. Nuclei Cont Excl

Run the series of steps.



e. Cytoplasmic Cont Excl

Run the series of steps.

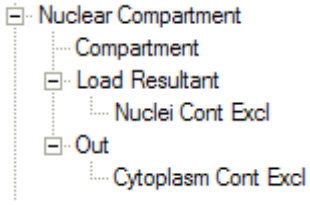


f. Nuclear compartment

Run the series of steps.

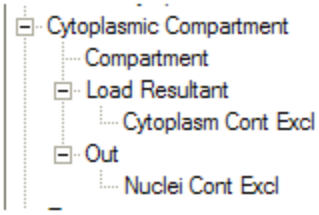
The **Out** procedure retains only those pixels from the current image that do not co-localize with pixels in the user selected **Resultant**. In this case, nuclear compartment pixels that co-localize with those of the Cytoplasmic compartment will be eliminated.

This is the area where we detect target protein pixel intensity in tumour nuclei.

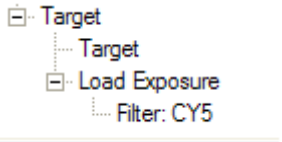


g. Cytoplasmic compartment

Run the series of steps. This is the area where we detect target protein pixel intensity in tumour cytoplam.



h. Target



NOTE: All step titles are suggestions only and can be changed. For example, it would be perfectly acceptable to name the target step `Estrogen Receptor` or after any other biomarker being used.

This completes the experiment setup.

AQUA® Scoring:

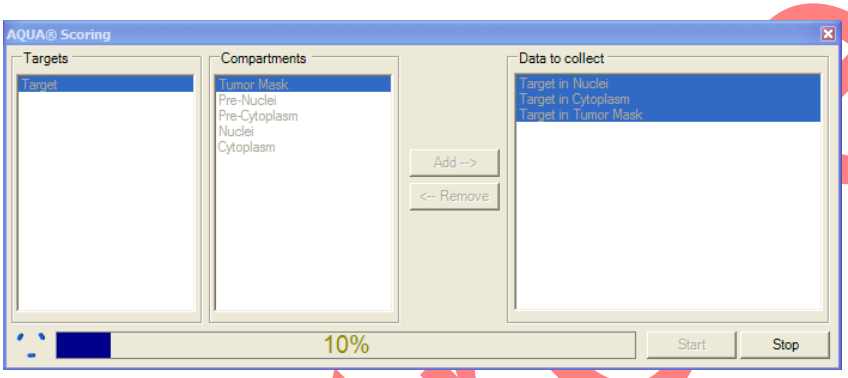
On the **Tools** menu, click **AQUA® Scoring**.

In the **Targets** pane of the **AQUA® Scoring** window, select Target.

In the **Compartments** pane of the **AQUA® Scoring** window, select the compartments in which the target will be quantified.

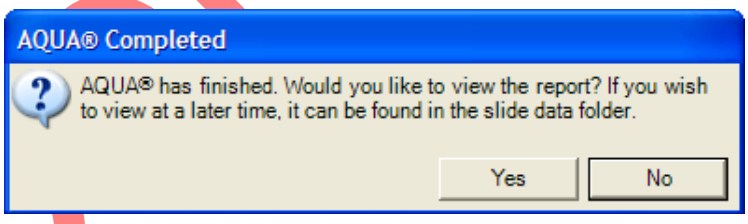
TIP: Hold down the **CTRL** button while clicking the target or compartment names to select multiple items at the same time.

Click the **Add-->** button to confirm the selection. To remove erroneous target\compartment combinations from the **Data to collect** pane, highlight the unwanted entries and click the **<--Remove** button



Click **Start** to begin the scoring process.

Although the analysis time varies depending on the number of steps as well as their complexity, an average experiment will take up to 30 seconds per spot. A progress bar shows the approximate completion level.

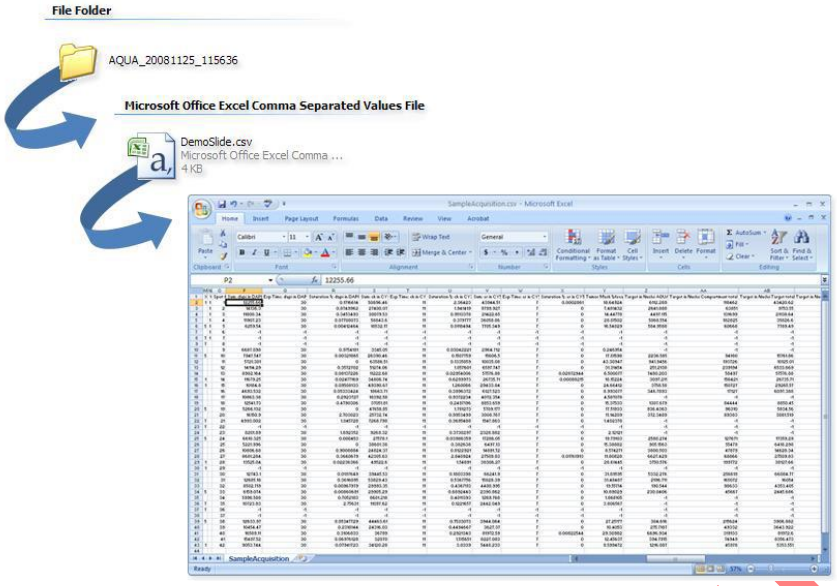


On the **AQUA® Completed** dialog, click **Yes** to view the **AQUAnalysis™ Scoring Results** report.

The report file is in the PDF format and will be displayed in the computer's default PDF viewer application.

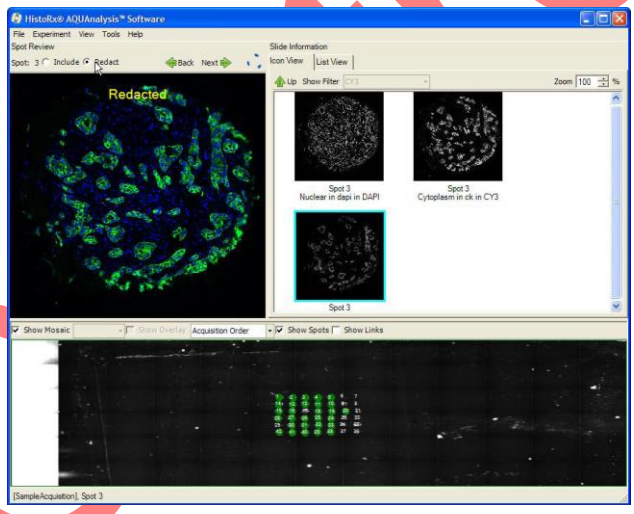
Close the PDF viewer application when finished with the report.

Retrieve Analysis Output File:



The analysis output data is automatically saved in the same directory as the **.tma** file, in a subfolder named according to **AQUA_YYYYMMDD_HHMMSS.**

The results file is in **CSV** (comma separated values) text format which can be opened by MS Excel as well as most statistical programs.



AQUAnalysis will now enter into the optional **Spot Review Mode**. In **Spot Review Mode**, the user can cycle through all analyzed images with the goal of flagging defective or faulty spots/fields-of-view.

5.0 Personal protection -

Howie coat must be worn at all times.

6.0 Training

Under no circumstances should this machine be operated by anyone who is not acquainted with it or has not been trained to use it.

7.0 Related documents –

7.1 Equipment manual

7.2 Equipment Maintenance Log

7.3 Equipment Maintenance Information sheet

7.4 Risk assessments – RA/GEN/038, RA/MH/002 and COSHH08

7.5 SOP - SASoM/EQUIP/024
Maintenance and replacement of EXFO lamp of the HistoRx ® PM-2000, AQUA

7.6 SOP - SASoM/EQUIP/025
Image acquisition using HistoRx® PM-2000, AQUA

8.0 Approval and sign off –

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Signature: Date:

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Position: Laboratory Manager

Signature: Date:

QA release by:

Name: Alex MacLellan

Position: QA Manager

Signature: Date: