

TECHNICAL BULLETIN

Using Excel Workbooks with AcycloPrime™ Kits and the EnVision™

IMPORTANT!

As part of their macro functions, these workbooks save files to the hard drive. If the macro fails to stop automatically after the number of saved “.xls” files equals the number of plates that were read, press the “Esc” key on the keyboard to manually stop the file generation process. Refer to section III for additional information.

I. General

Excel workbooks for the analysis of genotyping data generated with **AcycloPrime™** kits in either 96- or 384-well microplates and read using the PerkinElmer[®] Life and Analytical Sciences **EnVision™** plate reader are available for free download from the website www.snpscoring.com. The available workbooks are:

- A. EnVisionMacro_Excel96v7.0.xls**
- B. EnVisionMacro_Excel96v7.0ss.xls**
- C. EnVisionMacro_Excel384v7.0.xls**
- D. EnVisionMacro_Excel384v7.0ss.xls**
- E. EnVisionMacro_Excel384_4x96v7.0.xls**
- F. EnVisionMacro_Excel384_4x96v7.0ss.xls**

With all of the workbooks, data files are saved as “.xls” files that can be analyzed directly in **MS Excel**. In addition to that functionality, workbooks with “ss” in the name (B, D and F) also save “.txt” files for use with **SNPscorer™ Genotyping Analysis Software** (PerkinElmer Life and Analytical Sciences, Catalog Number ASP001). Workbooks A and B treat a 96-well plate as a single experiment, while workbooks C and D treat a 384-well plate as a single experiment. Workbooks E and F subdivide a 384-well plate into four 96-well assays using the template pattern shown on the **Plate Layout** page of the workbook. They allow 4 different 96-well assays to be performed on a single 384-well plate but to be analyzed individually.

II. Setup

- A. The setup of the **EnVision** hardware and software is described in the **Technical Bulletin “Using AcycloPrime Kits with the EnVision”**, which may be downloaded from the website www.snpscoring.com. Your EnVision should be running **v1.07** of the operating software. If necessary, contact **Technical Support** for a software upgrade. A licensed copy of **MS Excel** is not provided, but must be obtained by the user. The rest of this document assumes that you have already created a protocol file in the instrument software as described in the **Technical Bulletin**. For use with the workbooks, modifications needed to **Excel** settings and the **Event** page of the protocol are discussed below in **D**.
- B. Since some anti-virus software prevents downloading a macro-enabled file, the workbooks are provided as “.zip” files. Before use, they must be “unzipped” with software such as **WinZip**[®]. Download, unzip, and store the macro-enabled workbook(s) in any suitable folder on the hard disk, for example: **C:\EnVisionWorkbooks**.
- C. From within the **Wallac 2100 Manager** software, open the protocol by double-clicking on it, and select the specific wells to be read if not reading a full plate. The workbooks will function properly with various numbers of wells selected: from 1 - 96 (using a 96-well plate with workbook **A** or **B**) or from 1 - 384 (using a 384-well plate with workbook **B**, **C**, **E** or **F**). Any modifications to the protocol are saved automatically.
- D. Select one of the following options and modify the **Excel** settings and/or the **EnVision** protocol appropriately:

Option 1

After reading a plate on the **EnVision**, the **Wallac 2100 Manager** only generates and saves a “.csv” data file. That data file may be copied to another computer for subsequent analysis in an **Excel** workbook and/or by **SNPscorer** or some other method of choice. Labs with very high throughput may prefer this option, because the utilization of the computer to analyze data limits its ability to simultaneously read additional plates with the **EnVision**. For this option, there should be **NO** entries under **Event** on the **Output** tab of the protocol.

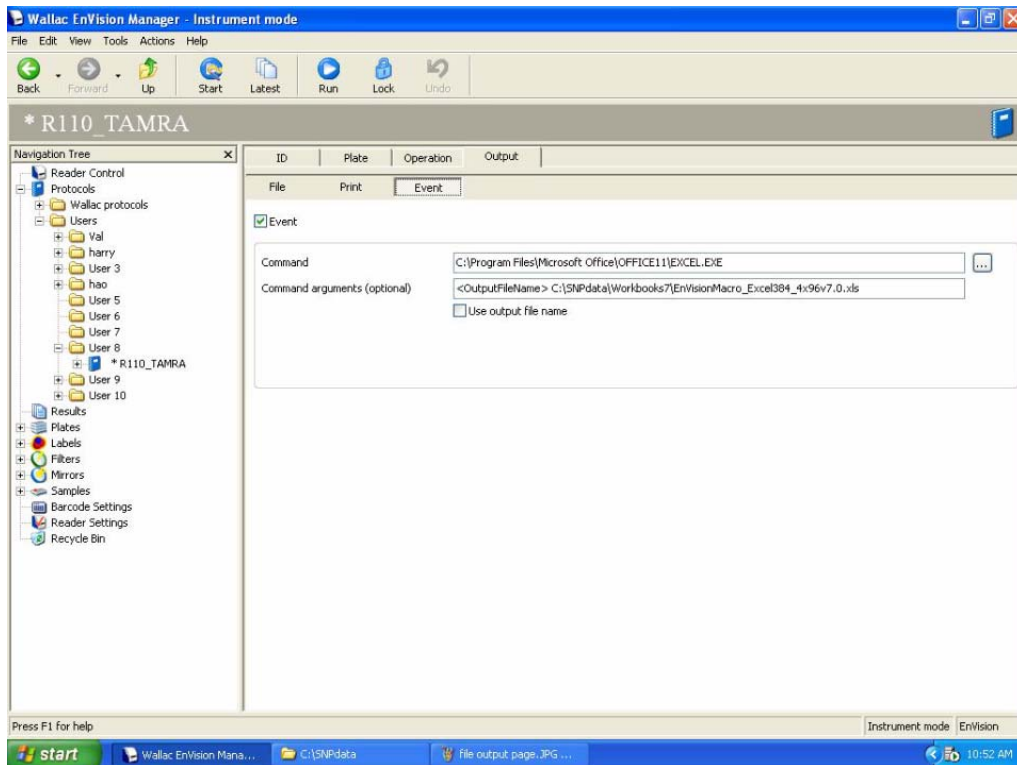
If using **Option 1**, open **Excel** and select **Tools\Macro\Security**. It is safest to set this to **Medium**. You will be prompted to enable macros to run whenever a workbook is opened.

Option 2

After reading a plate on the **EnVision**, the **Wallac 2100 Manager** software will generate and save a data file as in **Option 1**. Next, it will open **Excel**, then open a workbook and execute its macro instructions. The data for each plate read will be saved in a separate “.xls” file that can be used for genotyping in **Excel**. (Some workbooks will also save “.txt” files for genotyping with **SNPscorer** software.) After saving files, the data file, workbook and **Excel** will close.

If using **Option 2**, open **MS Excel**, and select **Tools\Macro\Security**. Set this to **Low** to enable macros to run without user intervention. (Otherwise the user will need to press a button to continue when the workbook opens.)

For all of this to happen automatically, **MS Excel** must be installed on the same computer as the **EnVision** operating software. It is also necessary to make certain entries under **Event** on the **Output** tab of the protocol as described in the **Technical Bulletin** mentioned previously and also shown in the following figure. Note that the box labeled **Command** contains the path to **MS Excel** and the box labeled **Command arguments** contains a reference to the name of the data file followed by a space and the path to the workbook being used.



E. Functional Testing of Installed Workbook(s)

For testing purposes, only a few selected wells need to be read, rather than an entire plate. Put the plate into the **EnVision** with well **A1** at the left rear. Select the protocol and click **Start** to test its functionality.

Option 1: Automatic execution of Excel workbook **not** enabled

1. If the settings are correct, a data file with the extension “.csv” is saved. After reading the plate, copy this file to a computer that has both **MS Excel** and the workbook installed on it and open the data as the **only** open workbook (no blank workbooks

either). On inspecting the first part of the file, you should see 16 rows and 24 columns of data for a 384-well plate or 8 rows and 12 columns of data for a 96-well plate.

2. A particular structure for this file is required for use with the workbooks. This structure is described in the table in section **III.B**. If necessary, modify the settings on the **Output/File** page of the **EnVision** software and re-read the plate. Repeat as necessary until the output “.csv” file has exactly the structure required. [If it is not possible to generate the proper data file by modifying instrument settings, you may need to upgrade the instrument software. Until that is done, you can analyze data with the workbook after manually pasting the plate data in the required locations as described in the box at the end of section **III.B**.]
3. Open **Excel** and select **Tools\Macro\Security**. If you set this to **Low**, the workbook macros will run without user intervention. However, since the macros will save files to your computer, it is safer to set this to **Medium**. That way, whenever a workbook is opened, you will be prompted to enable the macro to run and you can check to be sure that only one output .csv file is open in Excel at the time.

(Remember that the macro will attempt to operate on any other file that is open at the time it runs. If the open file is not an **EnVision** output .csv file with the proper structure, the macro can save large numbers of files to your computer as described in the box at the start of this document.)

4. If the structure of the “.csv” data file is correct and only that single file is open, open the workbook of interest. If prompted, agree to allow macros to run. You will see the macro executing, various worksheets being created, data being transferred and files being saved. Upon completion of the macro, all workbooks and **Excel** will close.
5. If using **Option 1**, skip to item 7.

Option 2: Automatic execution of Excel workbook enabled

6. After the plate is read, the **Wallac 2100 Manager** will save the data file. After a short delay, **Excel** and then the workbook will open, the macro instructions will execute and save an “.xls” file for each plate read. (Some workbooks also save a “.txt” file(s) for genotyping with **SNPscorer** software.) After files are saved, **Excel** and the workbook will close.
7. Open the folder designated for saving results and make sure that the expected new .xls and/or .txt file(s) have been created. Open the file(s) with the “.xls” extension and try to analyze the data as described in section **IV**. If the workbook produced “_Res_plt“#”.txt” files, try to analyze them using **SNPscorer** software.

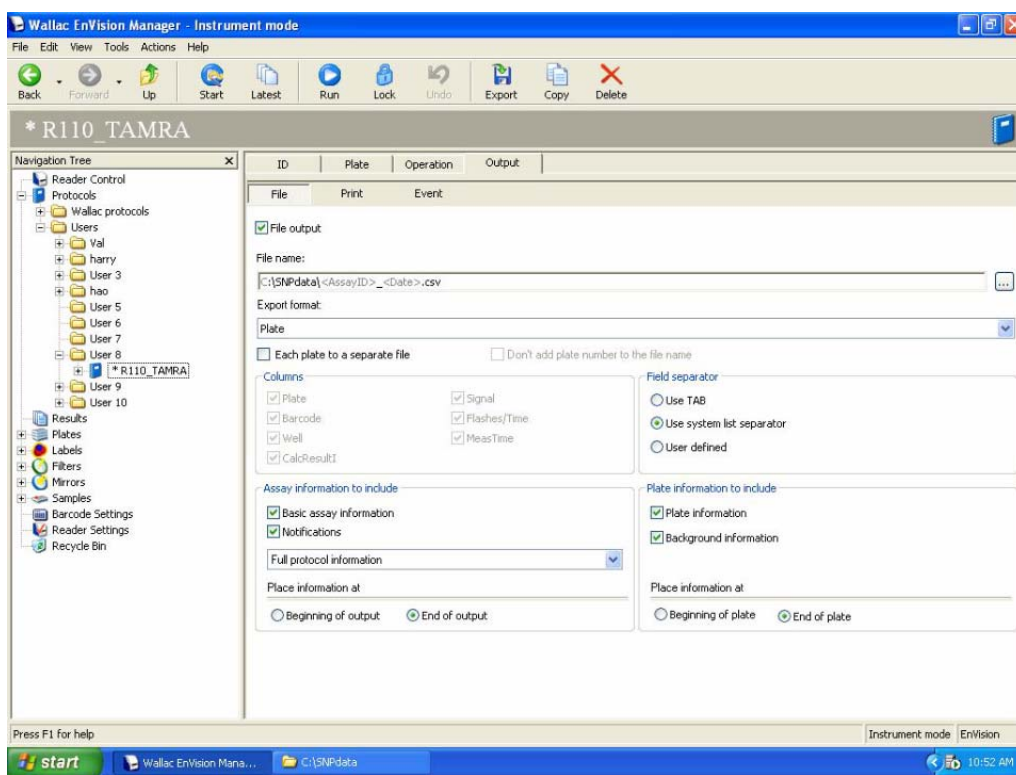
NOTE: The “Y” on the “Settings” page of the workbook should have been changed to an “N” when the “_Res_plt“#”.xls” data files were created. This change prevents the macro that saves additional files from running, so it is not necessary to avoid opening multiple such files for genotyping at the same time in **Excel**.

III. File Structure and Workbook Operations

A. End of the Stack

When using a plate stacker most efficiently, the **EnVision™** saves the data for all of the plates in the stack in a single output file. Therefore, part of the functionality of each workbook is to separate such data into a number of files so that each file contains the data representing a single plate. Thus the initial the workbook macro operation consists of opening the output data file and saving plate-sized “chunks” of data as individual files.

A stack does not contain a specific number of plates, so the macro must somehow determine when it has reached the end of the stack. To do this, the macro checks for the text string “**Basic assay information**”. This is the first line of information that is appended to the end of the data file by certain selections made in the **EnVision** software when it is set up according to the document “**Using AcycloPrime Kits with the EnVision**” (see www.snpscore.com). Since the presence of this text string in a specific location in the data file instructs the workbook to **stop** saving files, it is crucial that the output data file be set up properly in the EnVision software. The critical part of the protocol for determining the data file structure is the **Output/File** page, which should look as shown in the following figure.



NOTE: If this page is not set up properly, the text string “Basic assay information” will not be found in the anticipated location and the workbook will not stop saving files until the computer memory is full or until the “Esc” key is pushed. If the macro fails to stop automatically after the number of .xls files equals the number of plates in the stack, press the “Esc” key on the keyboard to manually stop the file generation process.

Because single plates are handled in an identical manner by the macro, it is not necessary to use a stacker to encounter this problem when using the workbooks. Occasionally, upgrades to the **EnVision** operating software have resulted in changes to the possible structure of the output data file. It is therefore necessary to be sure you use a workbook that utilizes a structure for your output data file that it is possible to generate using your instrument software. Current workbooks are **v7.0** and work with **EnVision** operating software **v1.07**. Additional information below will help you determine whether the workbooks can be used with your data files.

B. File Structure

The following table describes the structure required of the **EnVision** output data file (*.csv), showing the locations of important information within the file as a range of cells seen to contain data when the file is opened in **Excel**. Other information present in the file is not shown. **All** information must be in the indicated locations in order for the workbooks to function properly.

Contents of cells	384-well plates	96-well plates
R110 mP TAMRA mP R110 S R110 P TAMRA S TAMRA P	A1 – X16 A27 – X42 A53 – X68 A79 – X94 A105 – X120 A131 – X146	A1 – L8 A19 – L26 A37 – L44 A55 – L62 A73 – L80 A91 – L102
“Basic assay information”	A158, 314, 470, etc.	A110, 218, 326, etc.

When data for multiple 384-well plates are stored to a single file, the data for each plate occupies 156 rows. The string “**Basic assay information**” is located two rows after the data for the last plate. The macro inspects column A of rows 158, 314, 470, etc. for the text string: “**Basic assay information**” and when it is found, stops generating new files. For a 96-well plate, the data is in patterns of 108 rows and the macro inspects rows 110, 218, 326, etc. As described earlier, if the expected data pattern is **NOT** seen, the macro will continue generating output files indefinitely, or until stopped manually by pressing the **Esc** key.

What if your data file does not match the required structure?

You can still use the workbooks for data analysis by manually transferring the data as described below.

WARNING: Do not open a workbook and allow macros to run when another file is already open in **Excel**. The macro will attempt to operate on any open file and will cause **Excel** to start saving files as described in the box on page 1.

Open **Excel**. Make sure no files or workbooks are open. Under **Tools/Options/Macro/Security**, click on “**Medium**”. Then open the workbook you plan to use. When prompted, click on the button labeled “**Enable Macros**”. Because no data file is open when the macro tries to run, a “**Runtime error ‘9’:**” message will be generated. Simply click “**End**”. Open the “**Settings**” page of the workbook and change the “**Y**” in cell **C5** to “**N**”. This change will prevent the operation of the macro that saves files, but permit the operation of the genotyping macros. Save the workbook under a new name and use the modified workbook to analyze your data.

To use the workbook:

Open the **EnVision** data file (*.csv), copy all of the data and paste it into the workbook page labeled “**EnV multiplateData Pasted Here**”. Close the data file. You can then copy each of the six “plates” worth of data (R110 mP, TAMRA mP, R110 S, R110 P, TAMRA S and TAMRA P) for plate 1 from the “**EnV multiplateData Pasted Here**” sheet and paste it into the “**Single Plate Worksheet**” page using the locations shown in the table above as a guide. For convenience, the “**Single Plate Worksheet**” page has these locations marked in plate layout format with a “**0**” in each well position.

When all the data for the first plate have been transferred, save the file under a name that includes the number of the plate from which the data in the “**Single Plate Worksheet**” page was derived. Then transfer the information for the second plate in the file. Every time a complete new set of data is placed on the “**Single Plate Worksheet**” page, the workbook must be saved again, using a name containing the number or another identifier for the plate that was the source of the data. Be careful to use a unique name that will not overwrite the file that contains the data for a previously saved plate.

Since the rest of the workbook functions operate automatically after the data is correctly entered on the “**Single Plate Worksheet**” page, the saved files can be used for genotyping as described in the next section.

IV. Using the Workbooks for Genotyping

These instructions are supplied as a stand-alone document to avoid saving a copy of them with every set of results. However, the first page of each workbook contains basic information about how to use its genotyping functions. [Note that for the "4x96" workbooks, all the steps described in this section are performed on a single page (**SNP 1**, **SNP 2**, **SNP 3** or **SNP 4**) for each assay, not on separate pages as described below for the other workbooks.]

1. On the "**mP data**" sheet, enter the single letter base code for the **R110** (cell **D3**) and **TAMRA** (cell **E3**) **AcycloTerminators** used (**A**, **C**, **G** or **T**). Also on the "**mP data**" sheet, enter: **PLATE ID**, **RUN DATE**, **Operator** name and **Marker** information. If desired, additional information may be added to this page for record-keeping purposes. For example, it may be useful to add information about the primers and the cycling conditions here.
2. There are 2 graphs on the "**Scatter Plot**" sheet. If necessary, change the maximum and minimum values of one or both scales to zoom in appropriately so that the data clusters fill the graph space. This is accomplished by double-clicking on an axis to bring up the "**Format Axis**" window. It may be desirable to place any extreme outlying points (such as any empty wells) off the scale so that the clustering of the remaining points can be examined more easily. The simplest way to remove any unwanted points is to manually edit the raw mP data to remove them.

The scatter plot on the left is a "normal" graph of **TAMRA mP** vs **R110 mP**. The scatter plot on the right is a graph of the **TAMRA mP** vs **R (Intensity Ratio)**. **R** is defined as $(100)(\text{TAMRA Total Intensity})/(\text{R110 Total Intensity})$. For assays in which the **R110** signal is significantly quenched, a plot of **TAMRA mP** vs the **Intensity Ratio** will often permit calling the genotypes (see Xiao, M., Phong, A., Lum, K.L., Greene, R.A., Buzby, P.R. and Kwok, P-Y., "Role of excess inorganic pyrophosphate in primer-extension genotyping assays", *Genome Res.* (2004) 14, 1749-1755.) Either graph may be used to call the sample genotypes.

3. In each scatter plot, there are 3 ellipses in different colors. The green ellipse represents the **TAMRA homozygotes**, the blue ellipse represents the **R110 homozygotes** and the red ellipse represents the **heterozygotes**. **Negative** samples are shown in black.

Clicking on a colored ellipse will bring up a set of "handles" that allow moving and resizing the ellipse until it encloses all the points deemed to represent the corresponding genotype and none of the points that represent other genotypes or are uncertain. When all three clusters are located inside their corresponding ellipses on the preferred graph, click on the button on that graph labeled "**Assign Clusters**". The points inside each ellipse will turn a color that matches the ellipse. The points will also be labeled with the corresponding genotypes on the "**Genotypes**" page and in their well locations on the "**Well**" page. Points not located inside an ellipse remain black and are labeled "**Negative**". Similarly adjusting the ellipses and clicking on the "**Assign Clusters**" button on the other graph will use that data instead when assigning genotypes.

5. **SAVE** the completed worksheet under the desired final file name.

6. Inspect the resulting genotypes. For your convenience, a comparison tool is provided on the "**Summary**" sheet of some of the workbooks. If the genotypes for the samples in any wells are known in advance, type them into the "**EXPECTED**" column, "**E**". Column "**F**" automatically compares the experimentally-determined genotype in column "**B**" to the **EXPECTED** genotype. If they are the same, the result is "**TRUE**" and if different, the result is "**FALSE**". This is also a useful way to compare the results of two different experiments.

Examine those samples giving a result of "**FALSE**" and try to determine the possible cause(s). **NOTE:** Samples with a failed PCR reaction will always give a "**Negative**" result. If these samples have known genotypes inserted in the workbook, they will be reported as "**FALSE**" by the "**INSPECTOR**" function. **NOTE:** The "**INSPECTOR**" function has been removed from the "**4x96**" workbooks, which contain a different type of "**Summary**" sheet.

7. The user is free to edit any workbook to meet specific needs for personal use. The encoded macros and the cell-specific copy and analysis functions included are not protected other than by the basic **Excel** protection function located under **Tools\Protection**. Where necessary, just select **Unprotect sheet** or **Unprotect workbook**. Before performing any editing, make certain a master copy is archived for easy retrieval.

8. **SAVE YOUR WORK!**