TmTool™ Application User Guide

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Preface

The TmTool™ application analyzes fluorescent readings from melt experiments performed using Applied Biosystems Real-time PCR Instruments[1] and generates a table of the melting temperatures based on the Boltzmann equation. The tool is designed for melt profiles where the fluorescence readings rise with temperature.

The tool is a suite of scripts written in MATLAB and compiled into an executable. MATLAB is not required to run the tool, but MATLAB runtime environment is required.

The TmTool™ application was developed and tested on a Windows® XP 32 bit system with Service Pack 3. A minimum of 10G available disk space, 1G RAM available memory and a 2.4GHz CPU is recommended. TmTool™ application works best at a screen resolution of 1280 by 800 pixels.

Text conventions:

- Note – Indicates information that may be of interest or help but is not critical to the use of the TmTool.
- `Courier` font – Indicates code or command line code.
- *Italicized* text -- Used for emphasis.

1 Overview of the analysis tool

The TmTool™ application directly imports instrument run files from the StepOnePlus™ and the 7500 Fast Applied Biosystems Real-time PCR platforms. It can use text exports of multi-component data from the 7900 HT systems.

The TmTool™ application enables you to:

a. calculate a melting temperature (Tm) from their melt curve data using the Boltzmann equation,

b. designate reference samples,

c. compare samples to the reference samples by computing a ΔTm, defined as a shift in Tm value of their sample from the average Tm of reference samples,

d. visualize the melt curve of a sample with or without a reference curve in a picture format.

2 How to obtain the tool

Contact your local Life Technologies support representative for more information.

3 Install the MATLAB runtime environment

The following files are supplied as part of the installation:

```
MCRInstaller.exe
TmToolv1_0.ctf
TmToolv1_0.exe
UserGuidev1_0.pdf
```

[1] For further information on experiment run protocols, refer to TmTool™ Quick Set up Guide.

QuickSetupGuide.pdf

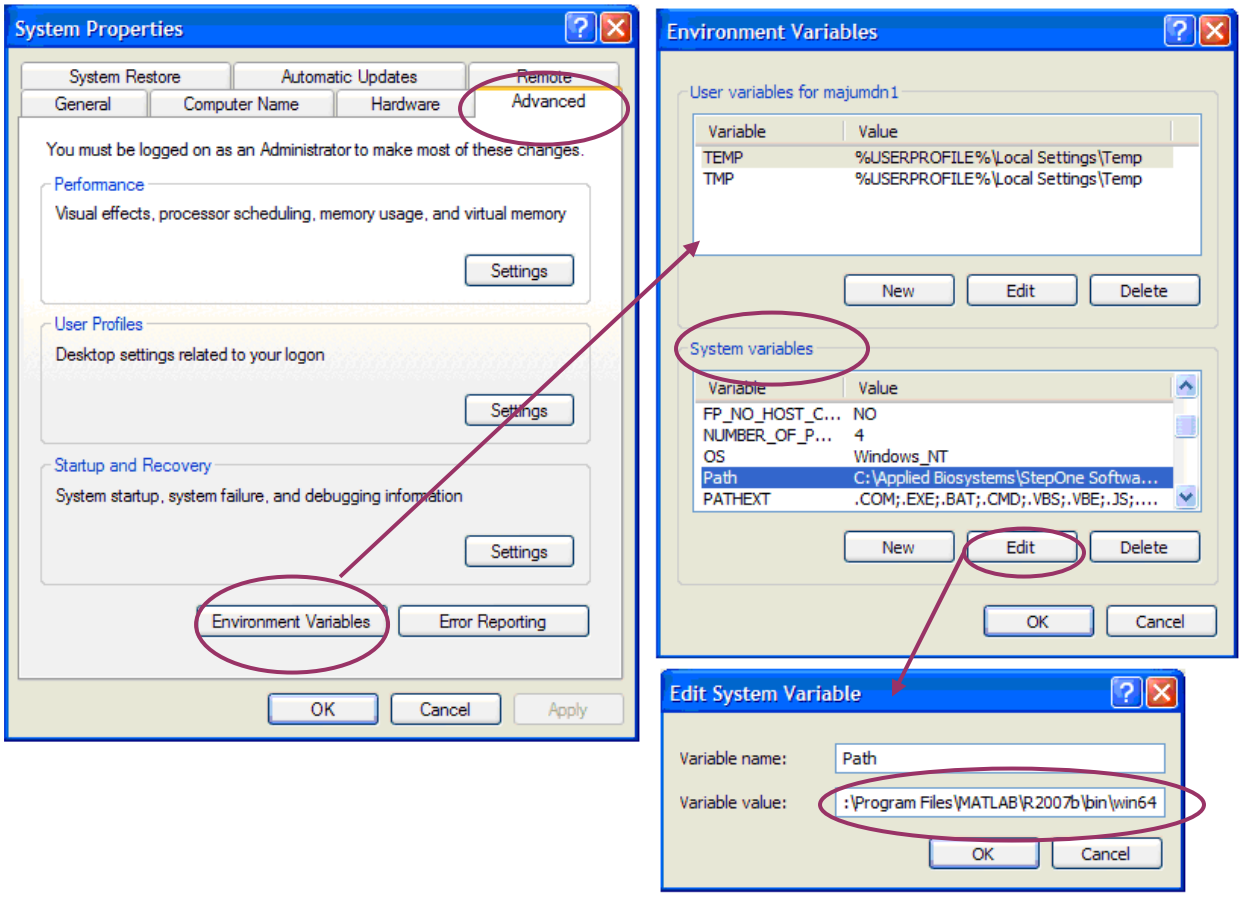
Note the installation does *not* create a shortcut to the TmTool™ application on the desktop.

1. Double click and install the MCRInstaller.exe to set up the MATLAB run time environment. The installer prompts you to choose the installation location. The default location is:
   C:\Program Files\MATLAB\MATLAB Component Runtime\

2. Add the following to the environment PATH variable:

   a. From the desktop, right-click "My Computer", then select "Properties".

   b. In the "System Properties" window, select the "Advanced" tab then click "Environment Variables".



   c. In the "System variables" section of the "Environment Variables" dialog box, select the "Path" variable, then click "Edit". Add or modify the path lines with the paths you wish the computer to access. Each different directory is separated with a semicolon. To access the tool, add:

`installDirectory\v77\bin\win32`
where `installDirectory` is the installation location you chose in step 1.

4 Working with TmTool™ Application

Run the experiment on the 7500 Fast, StepOnePlus™, or the 7900 HT systems following the guidelines in the QuickSetupGuide.pdf. Analyze the raw data using the instrument system software, then save the experiment file (*.eds or *.sds).

For the 7900 HT system, export the multi-component data from the ROX™ dye filter to a *.txt file to analyze the data using the TmTool™ application.

Note that no passive reference dye should be assigned to wells at the time of analysis.
At this point you can exit from the instrument software.

4.1 Starting the tool

To start the TmTool application, double click the `TmToolv1_0.exe`. This file is present in the `installationDirectory`. The first time this tool is run, it will bring up the end user license agreement. Click on `accept`. This will bring up the main interface as follows:

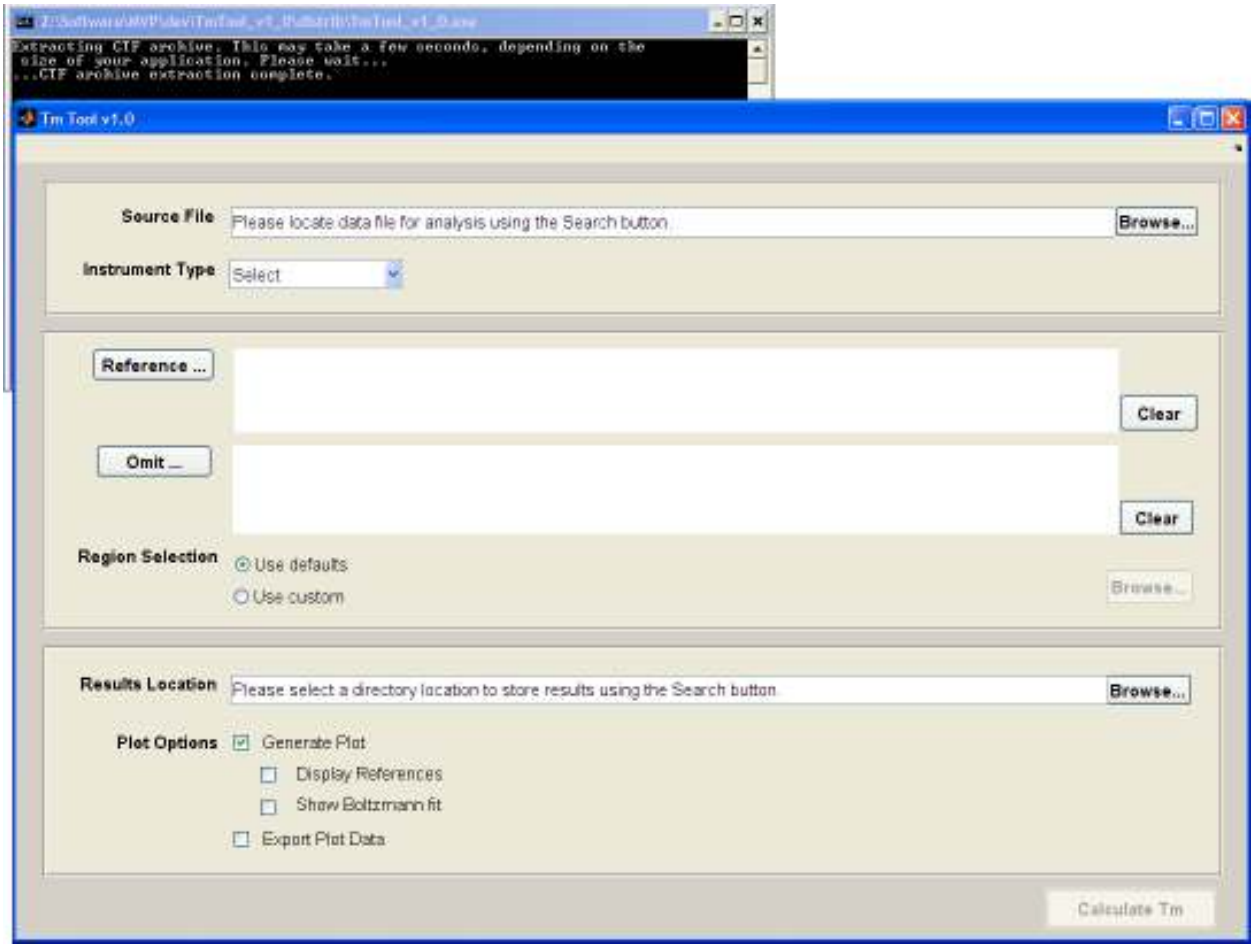
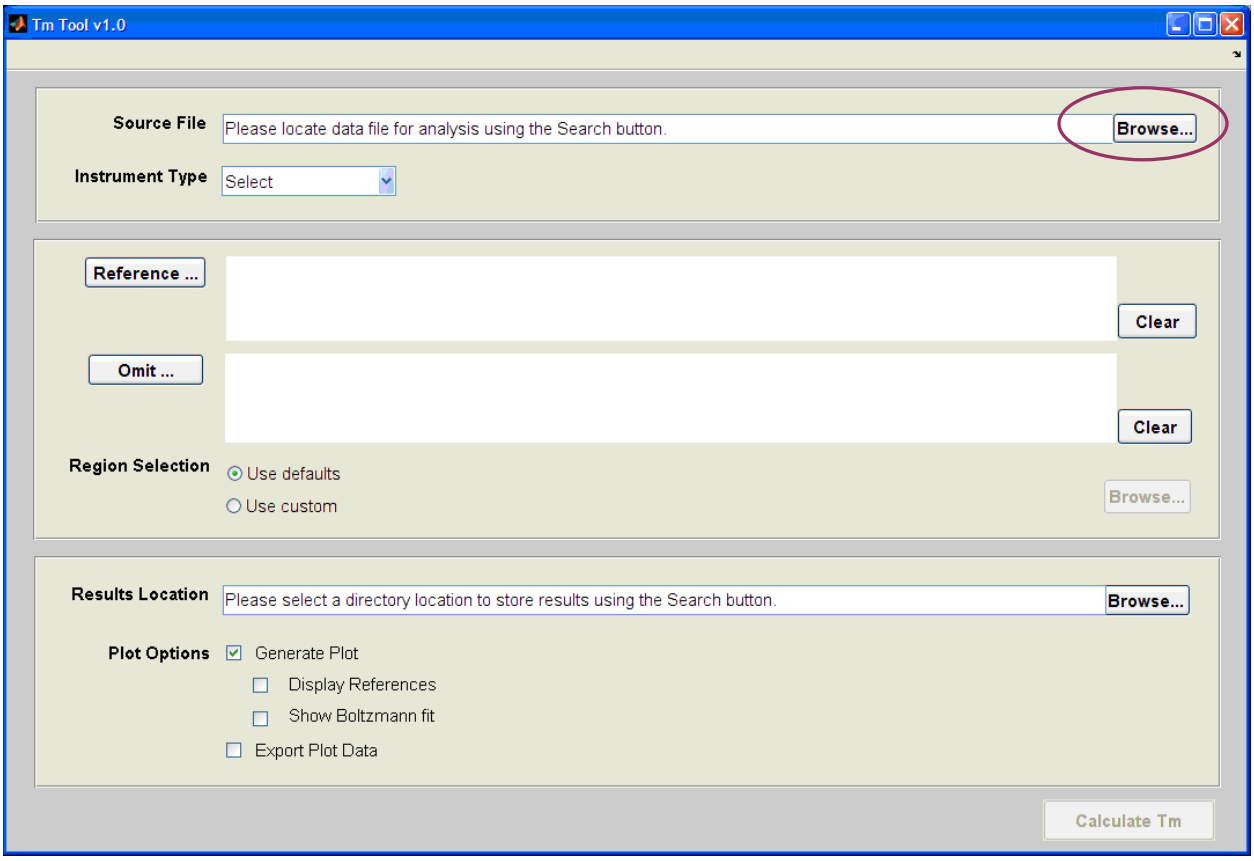


Figure 1: Main Interface of the TmTool™ Application

4.2 Importing a source file for analysis

The TmTool™ application can import data from *.eds files generated by the StepOnePlus™ and 7500 Fast Real-Time PCR Systems. For the 7900 HT system, the multi-component data from the ROX filter must be first exported from the SDS run file for use with the TmTool™ application.

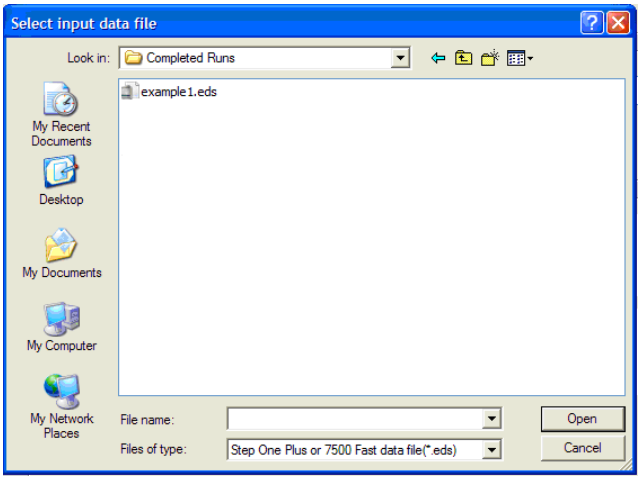
1. In the TmTool™ application, click `Browse` to navigate to the folder that contains the data files.



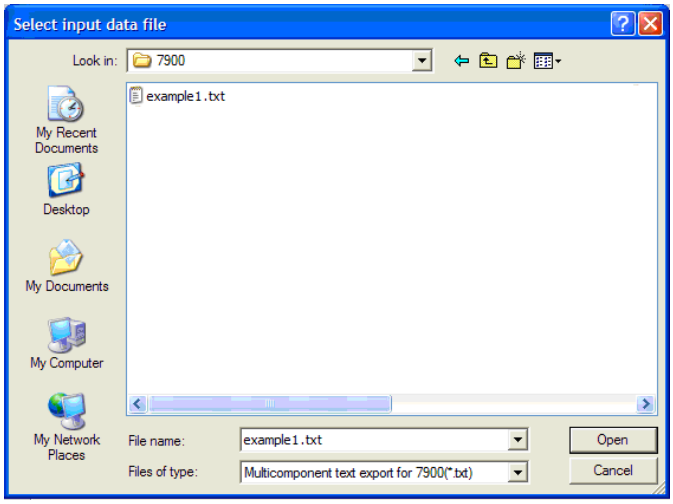
2. Select the data file, then click **Open:**

   By default, the *.eds files are listed.

   - To analyze files from the StepOnePlus™ or 7500 Fast systems, select an *.eds file, then click **Open:**



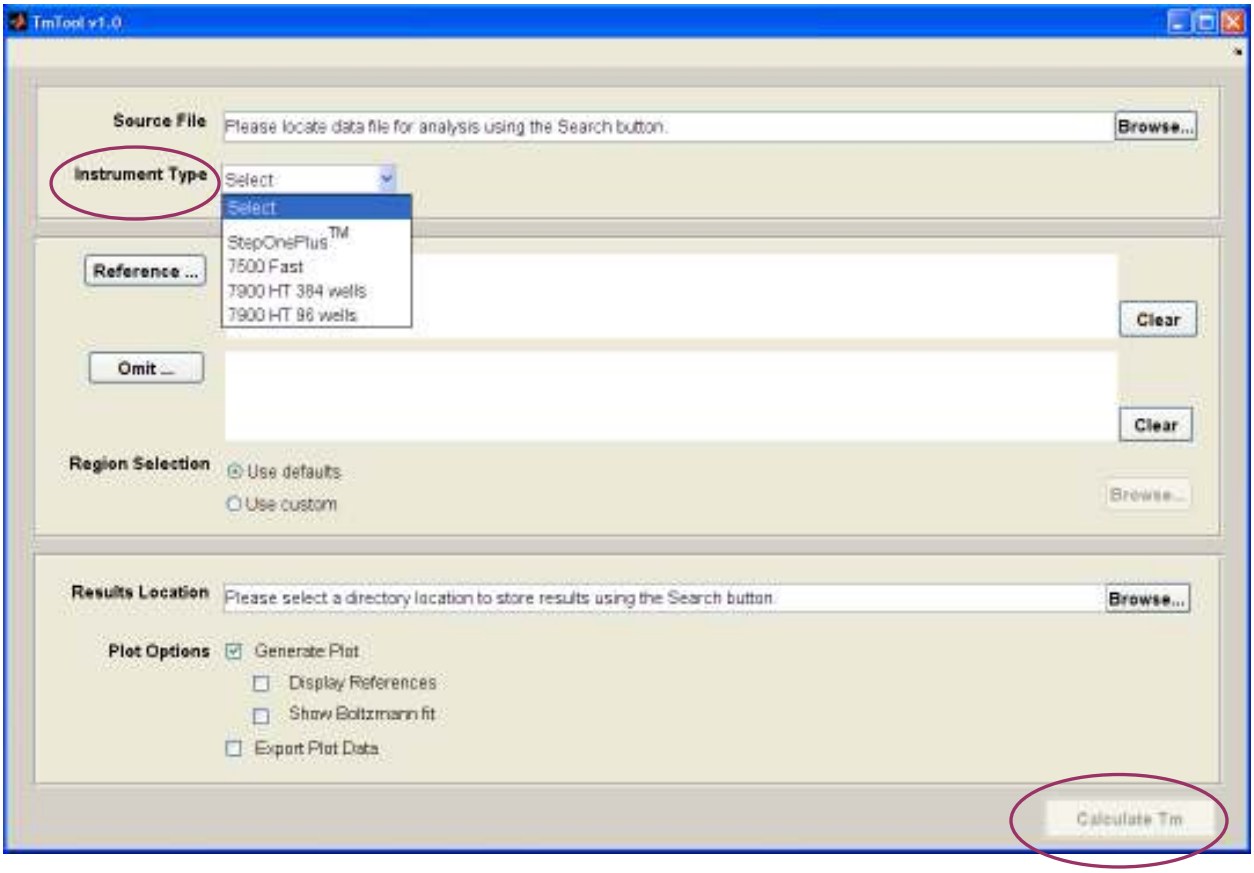
- To analyze a text export file from the 7900HT system, select from the "`Files of Type`" dropdown list, select a *.txt file, then click **Open**.



4.3 Selecting the instrument type

From the `Instrument Type` dropdown list, select the instrument type used to generate the data.

You need to select a source file and the instrument type to enable the `Reference`, `Omit`, and `Calculate Tm` buttons. The instrument type is validated with the source file at the time when the `Calculate Tm` button is pressed.

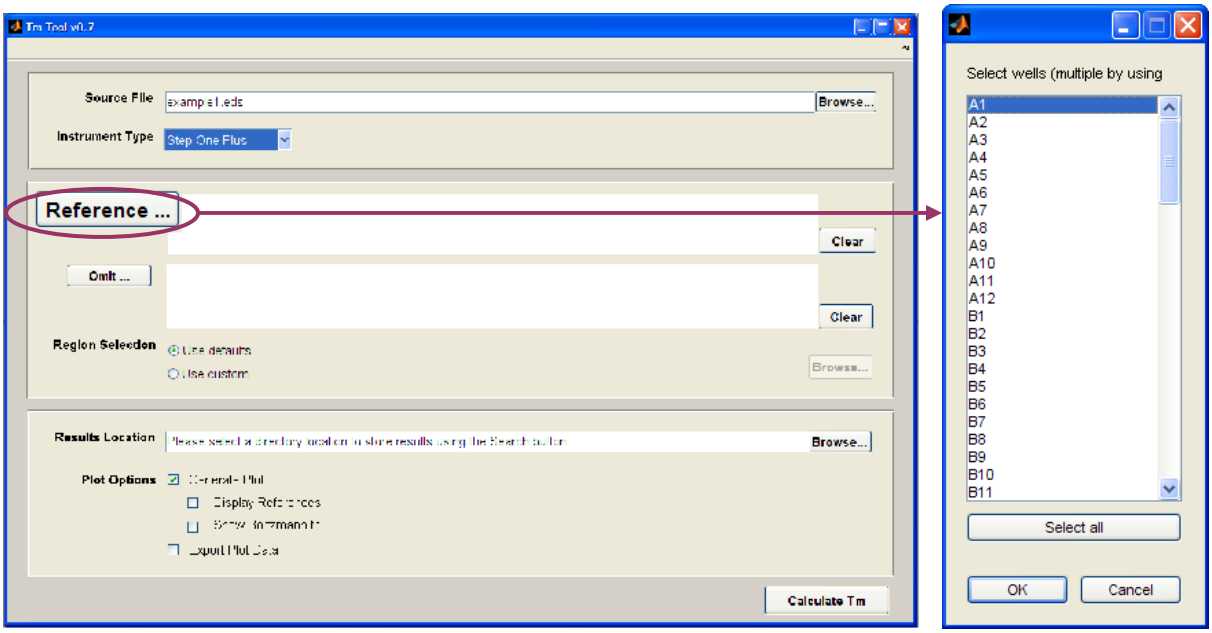


4.4 Identifying reference wells

If you designate one or more reference wells, the difference between the Tm of the sample in a given well and the average Tm value across the reference wells is calculated and reported as the ΔTm. You can choose to have the data from the reference wells superimposed on the data from the sample well in the well data plots that the tool generates.

To identify reference wells:

1. Click "`Reference`" to view a list of available wells. The list of available wells is dependent on the type of instrument you selected in the previous step. You need to select the instrument type to enable the "`Reference`" button.
2. Select the wells that contain reference samples.
    - Press the `ctrl` key while you click to select non-contiguous wells.
    - Press the `shift` key while you click to select contiguous wells.
    - Click `Select all` to select all wells in the list.
3. Press "`OK`" when you are finished identifying the reference wells.

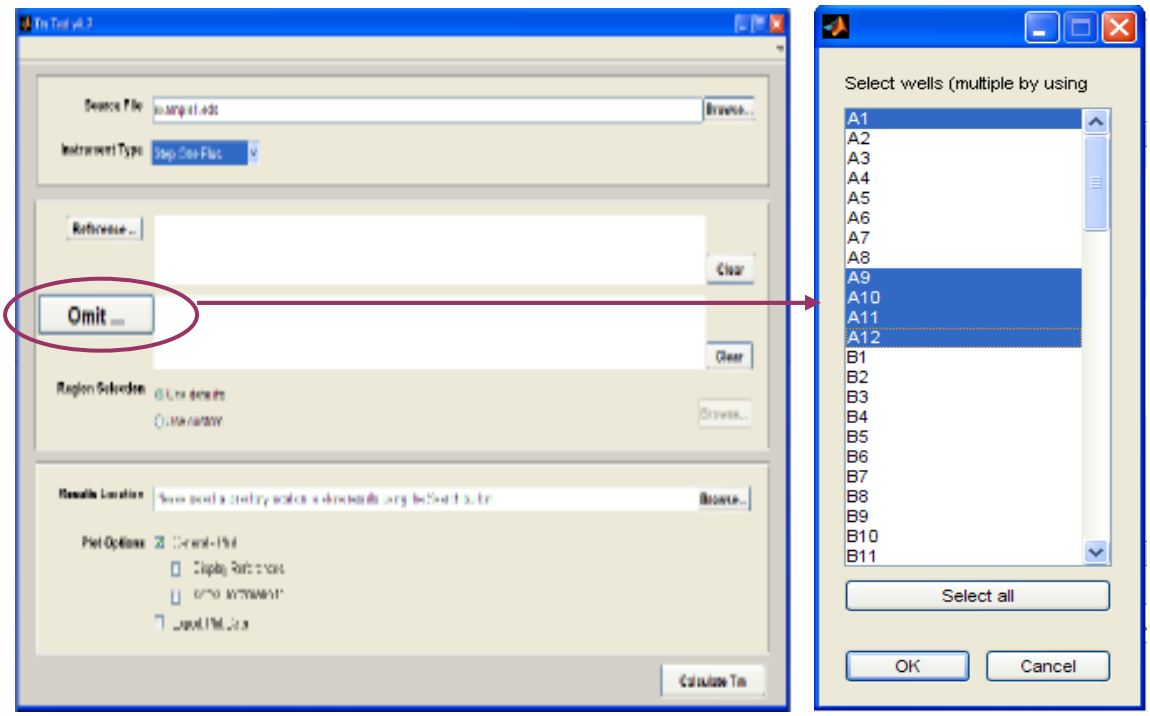

Tm Tool v0.7
Source File
example.eds
Browse...
Instrument Type
Step One Plus
Reference ...
Clear
Omit ...
Clear
Region Selection
Use defaults
Use custom
Browse...
Results Location
Browse...
Plot Options
Display References
Export Plot Data
Calculate Tm
Select wells (multiple by using
A1
A2
A3
A4
A5
A6
A7
A8
A9
A10
A11
A12
B1
B2
B3
B4
B5
B6
B7
B8
B9
B10
B11
Select all
OK
Cancel

4.5 Identifying wells to omit from analysis

You may want to omit certain wells from analysis. Figure 2 shows selection of wells A2 and A9 to A12 for omission.

To identify wells to omit from analysis:

1. Click `Omit` to view a list of available wells. The list of available wells is dependent on the type of instrument you selected in the previous step. You need to select the instrument type to enable the `Omit` button.
2. Select the wells to omit from analysis.
    - Press the `ctrl` key while you click to select non-contiguous wells.
    - Press the `shift` key while you click to select contiguous wells.
    - Click `Select all` to select all wells in the list.
3. Press `OK` when you are finished identifying the wells to omit.

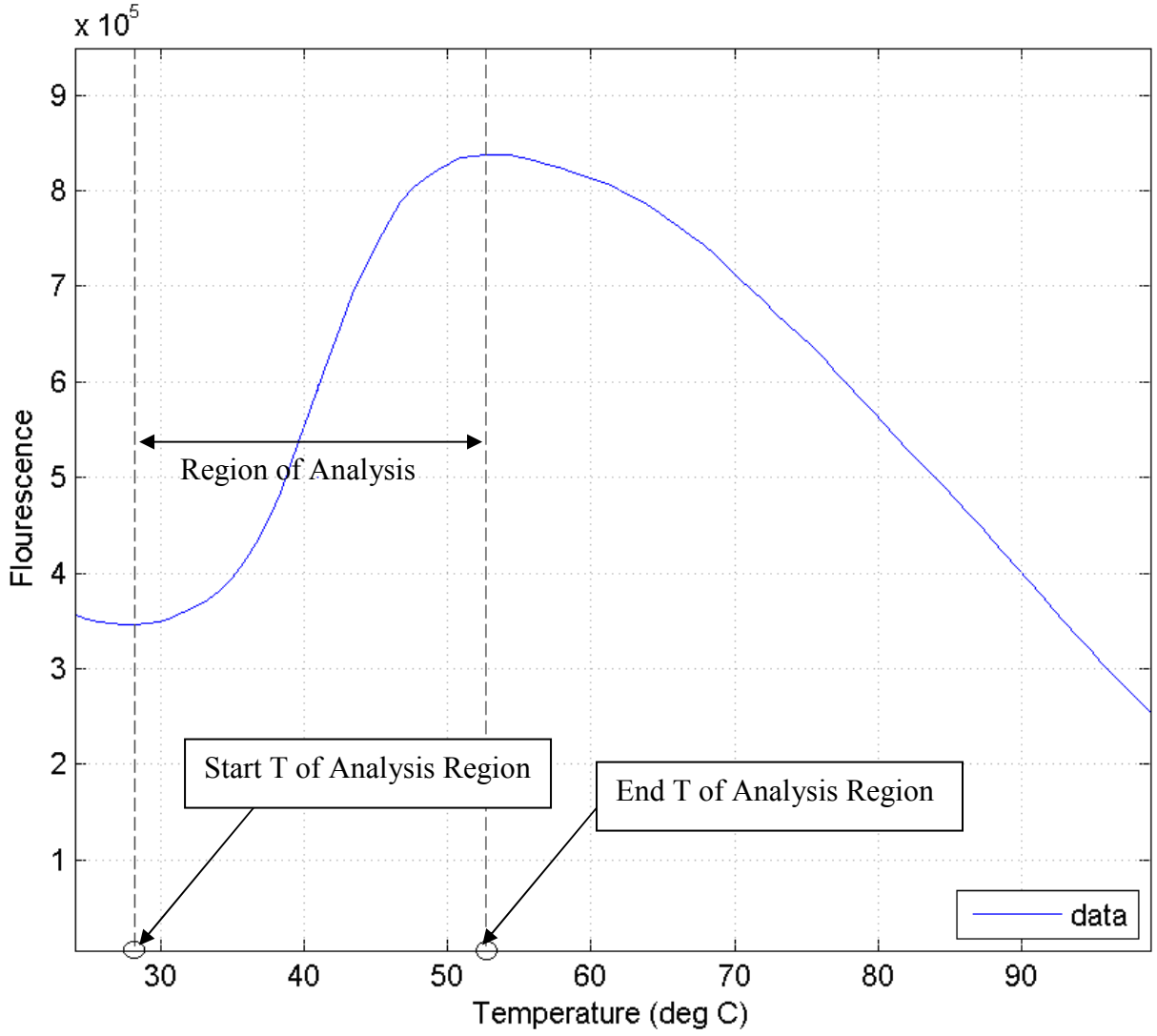


Note that if certain wells are selected for omission, they will not be available in the "`Reference...`" well selection window.

Also, note that the axis limits of the data plots per well are set using the range of fluorescence values from the set of non-omitted wells.

4.6 Region of analysis: default selection

The region of analysis is automatically chosen such that there is a continuous rise in fluorescence values over that temperature range. If there are multiple regions in the melt profile that meet this definition, the steepest of these regions is selected for analysis. Given below is a typical melt profile. The regions between the black dashed lines indicate the region of analysis.



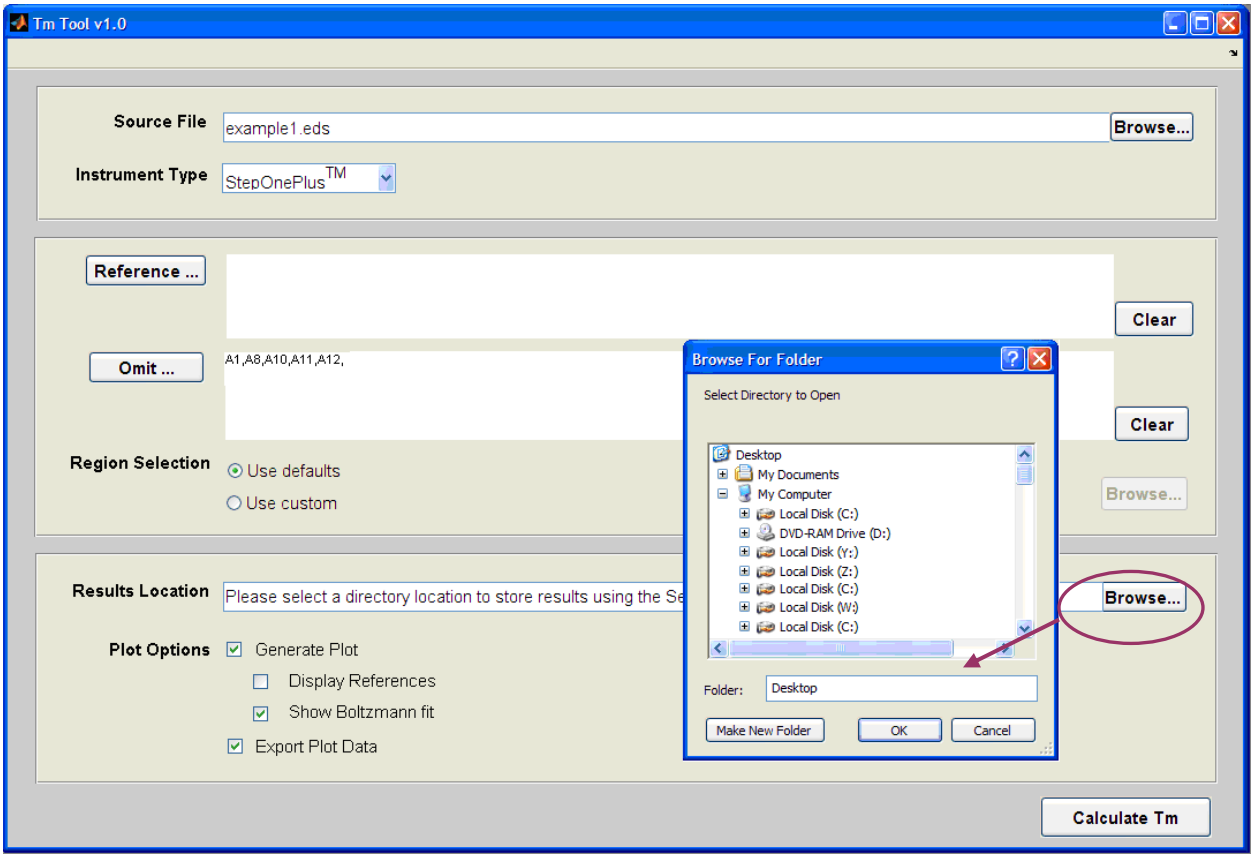
It is recommended that the default settings for region of analysis be used for the first analysis of every file.

Note that the Tm detection process is impacted by the appropriate selection of the region of analysis. For fluorescence profiles with multiple phases of melting, the ability to choose the region of analysis allows the flexibility of analyzing multiple regions of interest in the melt profile in separate runs of the TmTool™ application.

4.7 Choosing a location to store the results

From the TmTool™ application, you can select a directory location for storing analysis results. The TmTool™ creates a folder at that location with the same name as the name of the file given for analysis.

Note that if you do not select a location for storing the results, the default location for storing analysis results is the current working directory where the TmTool™ has been installed.



4.8 Selecting plot options

You can select plot options in the main window of the TmTool™ application.

- Select the `Generate Plot` checkbox to create a data plot for each well that is not omitted.
- Select the `Display References` checkbox if you designated reference wells (as per Section 4.4) and to superimpose reference well data on top of the plot of the data from the well (see Figure 2)
- Select the `Show Boltzmann Fit` checkbox to superimpose the fit data on the sample data from the well. The Boltzmann curve display can be used to visually check the quality of the calculated fit (see Figure 2).
- Select the `Export Plot Data` checkbox to create an output file of the raw data in a tab delimited format within the results location (see Figure 2).

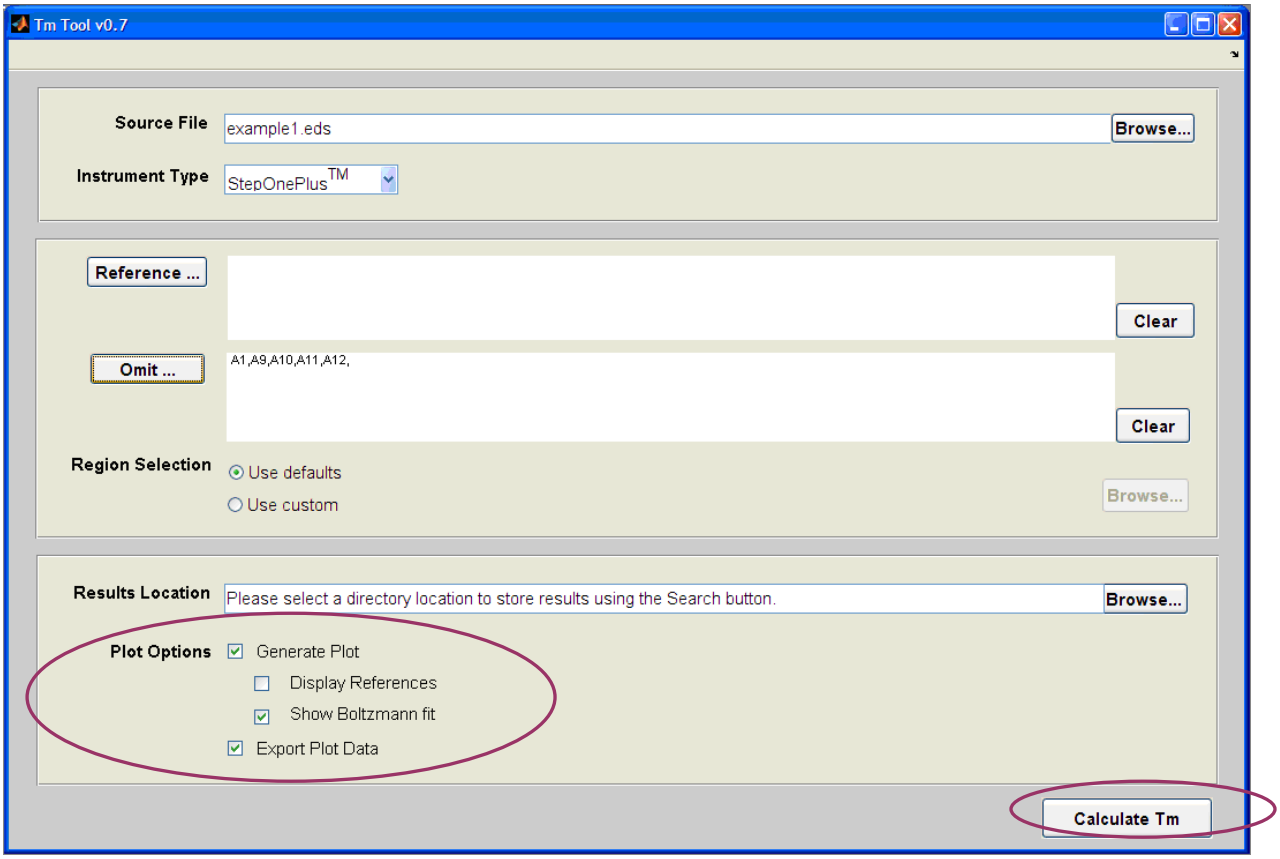
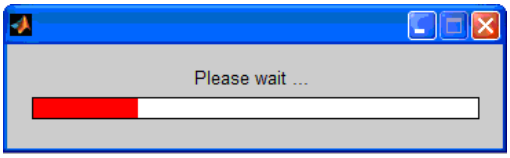


Figure 2: Plot Options

4.9 Calculate Tm

Press the `Calculate Tm` button (see Figure 2) to start calculating the Tm values. A progress bar appears to indicate that the processing is in progress.



When analysis is complete, the plots per well and the table of Tm values are generated. Results are stored in the folder indicated in the results location in section 4.7.

4.10 Reviewing the Results

4.10.1 Data Plots

After one round of execution, the main interface of the TmTool™ application as seen in Figure 1 is restored.

Plots of data per well are generated in the results location. An example data plot generated for well A2 is shown in Figure 3.

- The green line shows the Boltzmann fit produced by the tool.
- The dotted vertical lines delimit the region of analysis.
- The blue vertical line indicates the Tm value produced by the fitting.
- The blue line showing the melt curve is the fluorescence data from the sample in that well.
- The position where warnings or error flags appear, if any are generated, is also indicated in the figure. This example has no flags associated with the result.

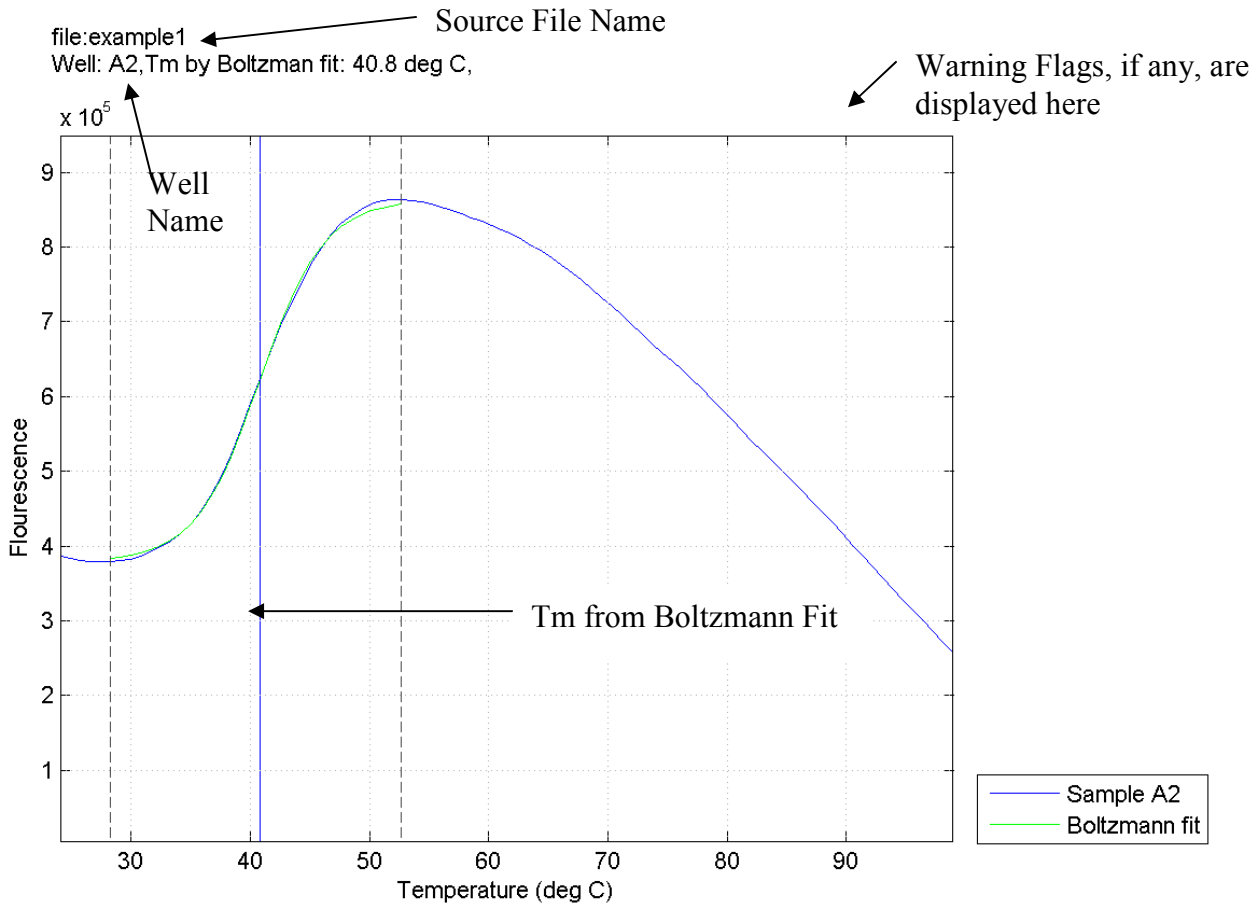


Figure 3: A sample plot of data from a well

4.10.2 Table of Tm Values

The TmTool™ application generates a table of melting point temperatures for each well. The results are stored in a tab-delimited file with the naming convention "`<filename>_Tm`" where <filename> is the name of the input data file. This table has the following columns:

- Wells: well name
- Tm Boltzmann (deg C): Tm from fitting data in the region of analysis to the Boltzmann Equation.
- Delta Tm: If reference wells were indicated, the difference between Tm from the given well and the average Tm of the reference wells is reported as the delta Tm.
- Warning: Warning/error flags that may apply to the result. For a complete list of error/warning messages see Table 1.
- Start T for Boltzmann Fit: Start temperature of the region of analysis
- End T for Boltzmann Fit: End temperature of the region of analysis

The output file "`<filename>_Tm.txt`" can be opened using Microsoft Excel software. An example of the output file is shown in Figure 4.

	A	B	C	D	E	F
1	Wells	Tm Boltzmann (deg C)	Delta Tm	Warning	Start T for Boltzmann fit (deg C)	End T for Boltzmann fit (deg C)
2	A1					
3	A2	40.8			28.25	52.66
4	A3	40.8			29.09	52.66
5	A4	40.3			28.25	51.82
6	A5	41.2			29.09	53.5
7	A6	40.9			29.09	53.5
8	A7	40.6			29.09	52.66
9	A8	40.4			28.25	52.66
10	A9					
11	A10					
12	A11					
13	A12					
14	B1	41.1			28.25	53.5
15	B2	40.9			28.25	52.66
16	B3	40.6			28.25	53.5
17	B4	40.3			28.25	52.66
18	B5	41.2			28.25	54.34
19	B6	41.1			28.25	54.34
20	B7	40.7			28.25	54.34
21	B8	40.5			28.25	54.34
22	B9	92.1		Low Signa	82.11	96.42
23	B10	94.3		Low Signa	85.48	98.95
24	B11	94.3		Low Signa	89.69	96.42
25	B12			Undetermined		
26	C1	46.7			24.05	53.5
27	C2	46.7			30.78	54.34

Figure 4: Table <filename>_Tm.txt

Entries corresponding to omitted wells are left blank in the <filename>_Tm.txt. In Figure 4, wells A1 and A9 to A12 were omitted from analysis.

For quality purposes, it is recommended that you review the data plots for all Tm values generated by the tool.

4.10.3 Table of Region of Analysis

The TmTool™ application generates a tab-delimited text file called `<filename>_AnalysisRegion.txt`. This table has three columns:

- Wells: well name
- Start T for Boltzmann Fit: Start temperature of the region of analysis
- End T for Boltzmann Fit: End temperature of the region of analysis

The <filename>_AnalysisRegion.txt can be opened using Microsoft Excel® software. An example is shown in Figure 5. The use of this file is explained in section 4.11.

	A	B	C
1	Wells	Start T for Boltzmann fit (deg C)	End T for Boltzmann fit (deg C)
2	A1		
3	A2	28.25	52.66
4	A3	29.09	52.66
5	A4	28.25	51.82
6	A5	29.09	53.5
7	A6	29.09	53.5
8	A7	29.09	52.66
9	A8	28.25	52.66
10	A9		

Figure 5: Table <filename>_AnalysisRegion.txt

4.10.4 Table of Plotted Melt Curve Data (optional)

The TmTool™ application generates a tab-delimited text file called as `<filename>_SmoothedMeltCurves.txt`. This table has the plotted data for each well along the rows.

4.11 Defining Custom Region of Analysis for wells where necessary

In some instances the default region of selection for analysis may not be appropriate to the data, as deemed by the closeness of fit of the Boltzmann fitting line that can be superimposed over the sample data. To define a custom region of analysis:

1. Locate the "`<filename>_ AnalysisRegion.txt`" in the results location and open it with Microsoft Excel software. Edit the start and end temperature for the region of analysis of each well as required (see Figure 6). For example, for well C1, the tool had selected 24.05 to 53.5, but the region was redefined from 35 to 53.5° C.

Formatte
Roman, 12
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Roman, 12
grammar
Deleted:

	A	B	C	D	E
1	Wells	Start T for Boltzmann fit (deg C)	End T for Boltzmann fit (deg C)		
2	A1				
3	A2	28.25	52.66		
4	A3	29.09	52.66		
5	A4	28.25	51.82		
6	A5	29.09	53.5		
7	A6	29.09	53.5		
8	A7	29.09	52.66		
9	A8	28.25	52.66		
10	A9				
11	A10				
12	A11				
13	A12				
14	B1	28.25	53.5		
15	B2	28.25	52.66		
16	B3	28.25	53.5		
17	B4	28.25	52.66		
18	B5	28.25	54.34		
19	B6	28.25	54.34		
20	B7	28.25	54.34		
21	B8	28.25	54.34		
22	B9	82.11	96.42		
23	B10	85.48	98.95		
24	B11	89.69	96.42		
25	B12				
26	C1	35	53.5		
27	C2	30.78	54.34		

Edit start temperature for region of analysis

Edit end temperature for region of analysis

Figure 6: Define Custom Range of Temperature for Analysis

2. Save this edited version of the analysis region file anywhere. Name the file as `<any text>_AnalysisRegion.txt`.

Note that the file containing the custom region of analyses must be named as "`*_AnalysisRegion.txt`" for the TmTool™ application to locate the file in the next step. "`*`" in the file name can stand for any text.

3. Select the "`Use Custom`" option from the TmTool™ application as indicated in Figure 7. The "`Browse`" button is activated. Click `Browse` to navigate to the location where you saved the analysis region file (step 2).

4. Run the analysis again using the "`Calculate Tm`" button.

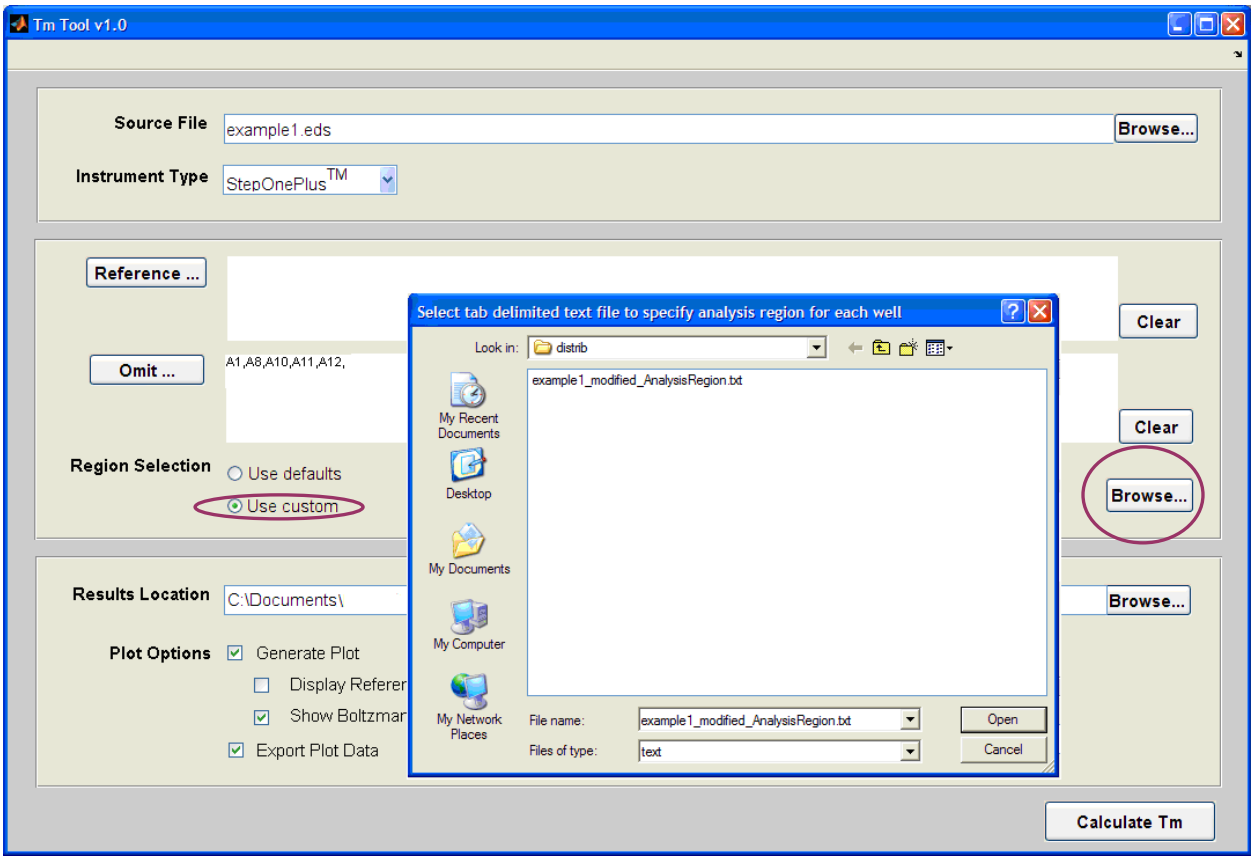

Figure 7: Select file with custom analysis regions defined

If the results location was not changed, a new directory called `<filename> 2` is created and the new results files are saved there. Figure 8 shows the data plot from well C1 with the region of analysis as defined in the previous steps.

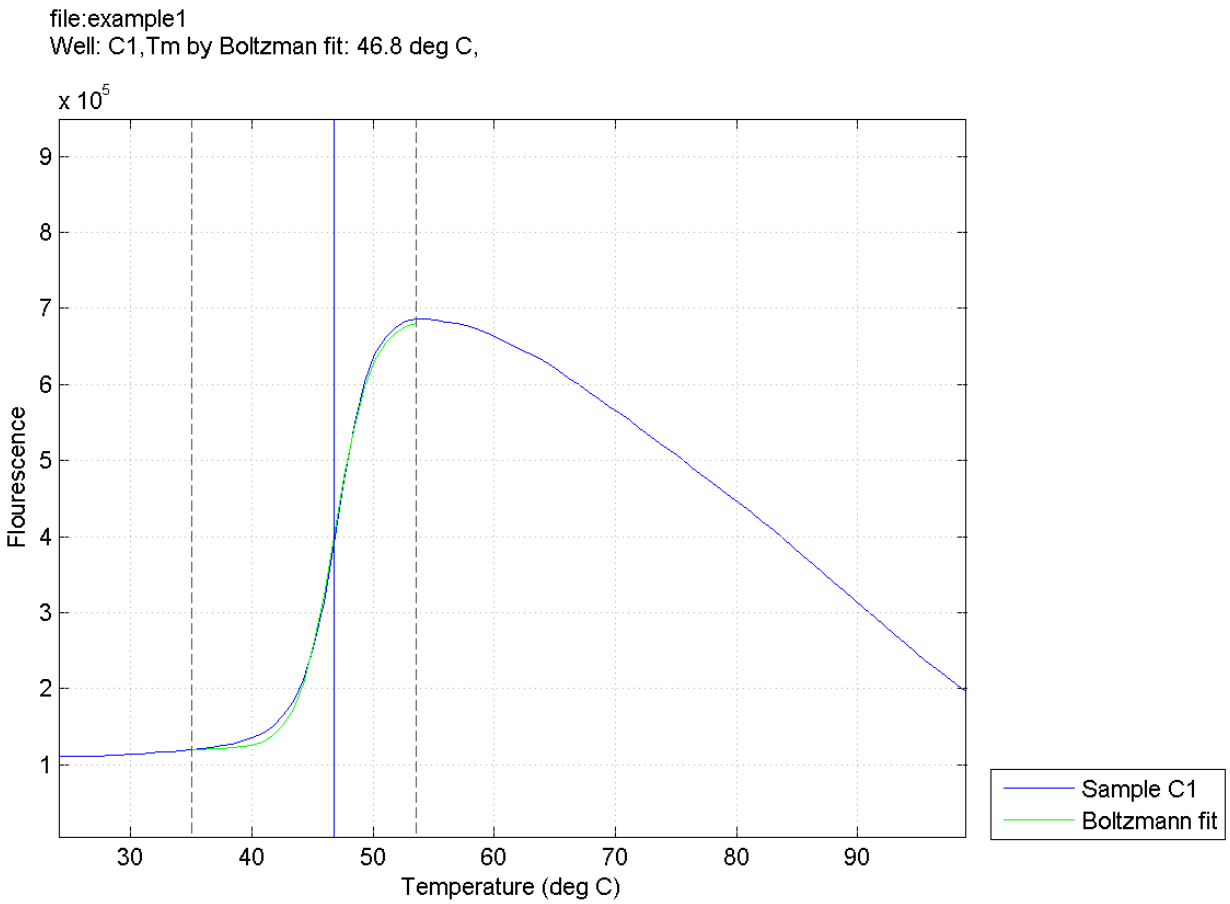

Figure 8: Well C1 with custom region of analysis

4.12 Designating Reference Wells, generating ΔTm and Plots with References

It is also possible to estimate the degrees of shift in Tm values between all the wells and wells C1 and C2.

1. Import a .eds file (see section 4.2).
2. Select the instrument type (see section 4.3).
3. Designate reference wells (see section 4.4).
4. Run the tool with the plot option `Display References` checkbox selected.
5. Use custom region of analysis defined in the `<filename>_AnalysisRegion.txt`. The analysis settings for this scenario are shown in Figure 9.

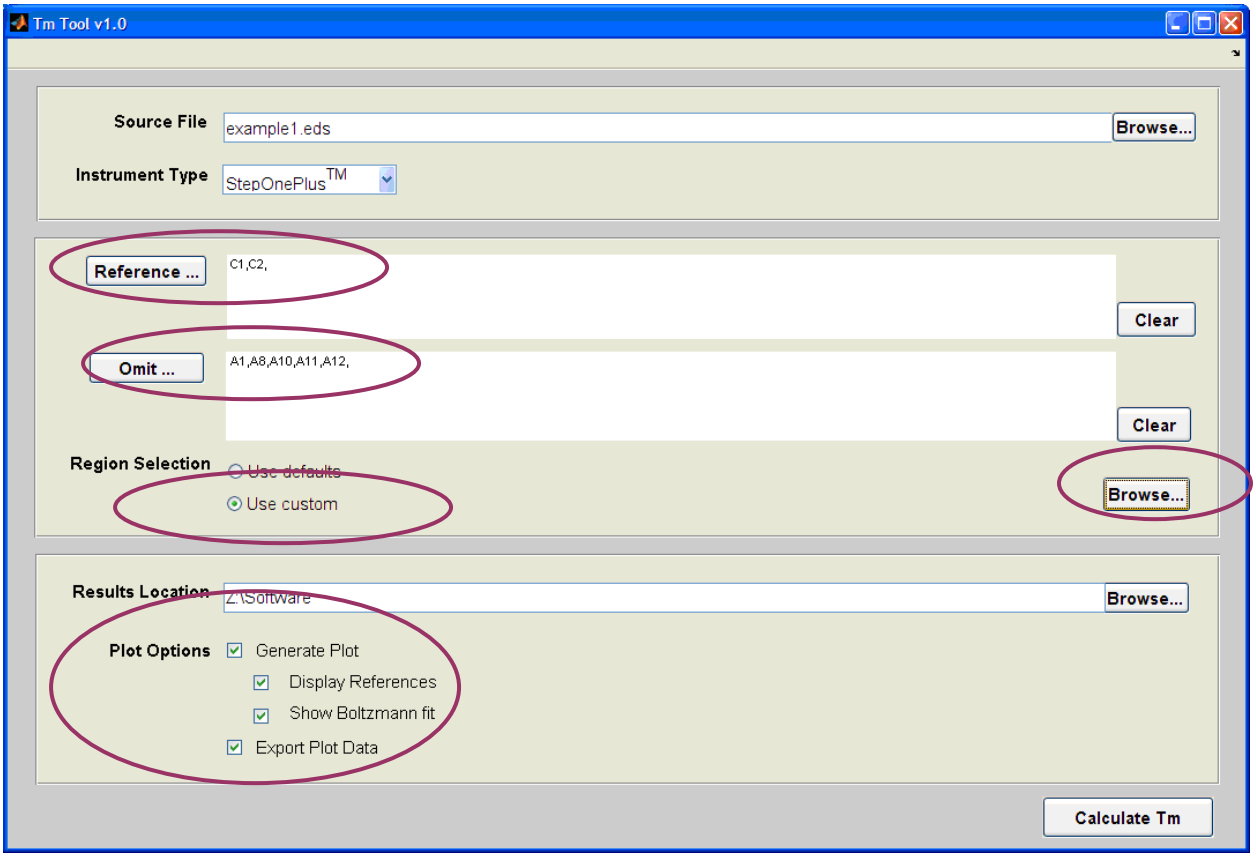

Figure 9: Using reference wells to generate ΔTm

An example of the plot generated with "`Display References`" selected is given in Figure 10. A ΔTm of 5.9 degrees C is observed between the average Tm of the reference wells and the Tm generated from data in well A3.

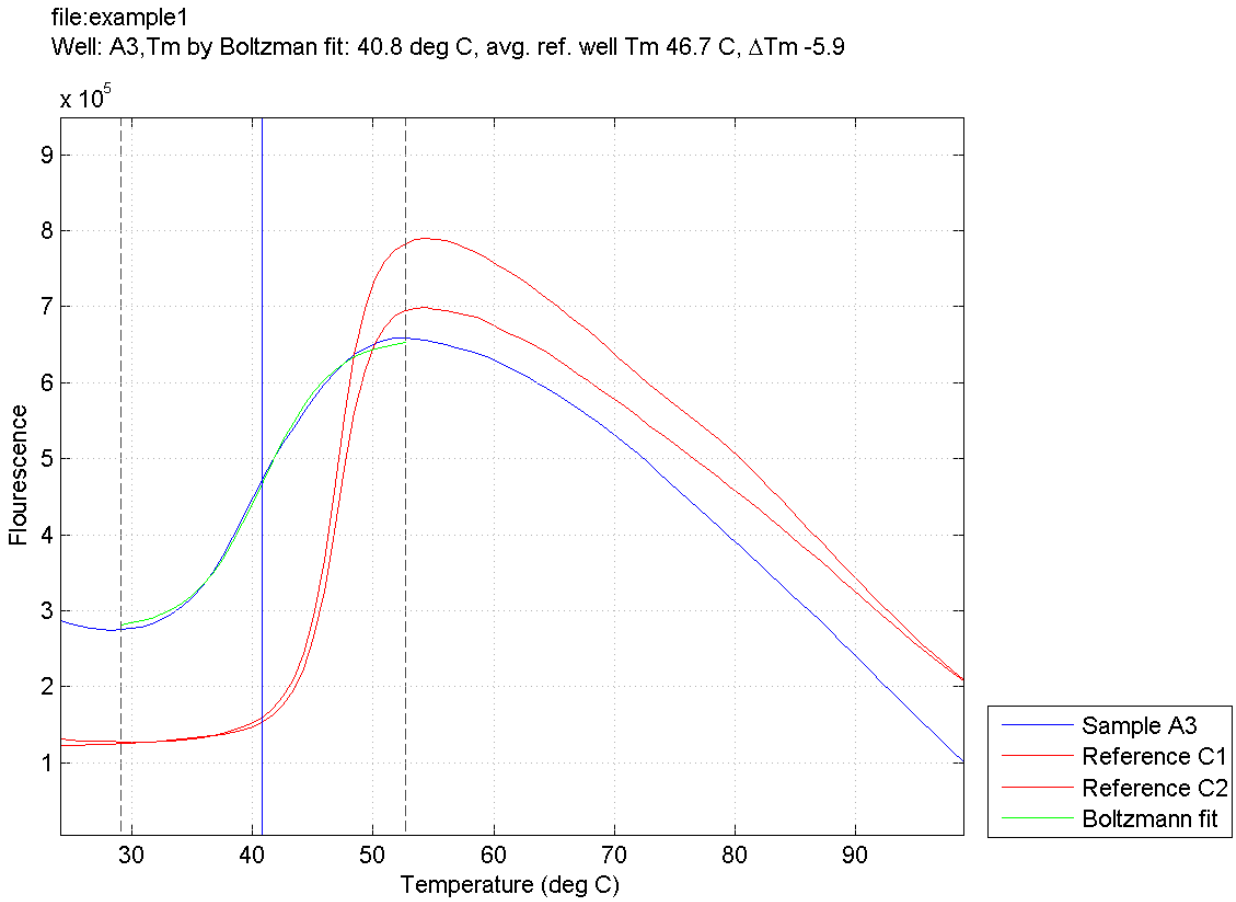

Figure 10: A sample plot of data from a well with reference wells

The result file `<filename>_Tm.txt` now has the column ΔTm populated. If you open this file using Microsoft Excel software, you can sort the wells by the ΔTm value to identify the well with the largest shift in Tm. The buffer condition for that well can then be identified as the buffer in which the sample proved to be maximally stable.

5 Troubleshooting

- Observation: The Microsoft Excel software has difficulty with opening the tab-delimited result files
  Possible cause: The path and file name combinations of the tab delimited result files may exceed 270 characters.
  Solution: Rename the tab-delimited results files, rename the file using a shorter name and then use MS Excel software to open it.

- Observation: The TmTool™ application is not able to locate the file where you saved the modified regions of analysis.

Possible cause: The path and file name combinations of the tab delimited result files may exceed 270 characters. Or, you did not save the file using (`*_AnalysisRegion.txt`) as the naming convention.
Solution: Rename the file with the analysis regions as `*AnalysisRegion.txt` file where the path and filename combination is under 270 characters.

- Observation: If there are multiple melt phases (Figure 11), the auto selected region of analysis selects the entire region over which a rise in fluorescence value was observed.
  Possible cause: The tool is programmed to select the *largest* contiguous region of rise in fluorescence until the point where the slope becomes negative.
  Solution: If this is not the desired behavior, it is possible to define a different region of analysis following the instructions in section 4.11.

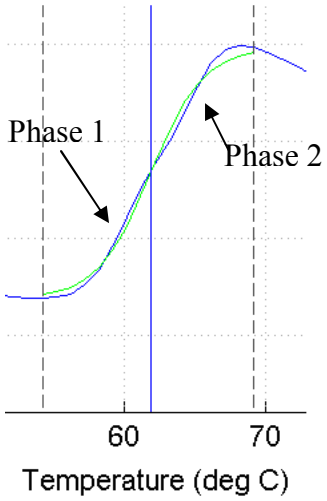

Figure 11: Multiple phases of melt, or, initial melt phase slowly rising

- Observation: If there are two melt phases, with one rising to a higher magnitude than the other (Figure 12), the auto selected region of analysis will still select the region of the steeper rise, even if it has a smaller magnitude.
  Possible cause: The tool is programmed to select the *steepest* contiguous region of rise in fluorescence.
  Solution: If this is not the desired behavior, it is possible to define a different region of analysis following the instructions in section 4.11.

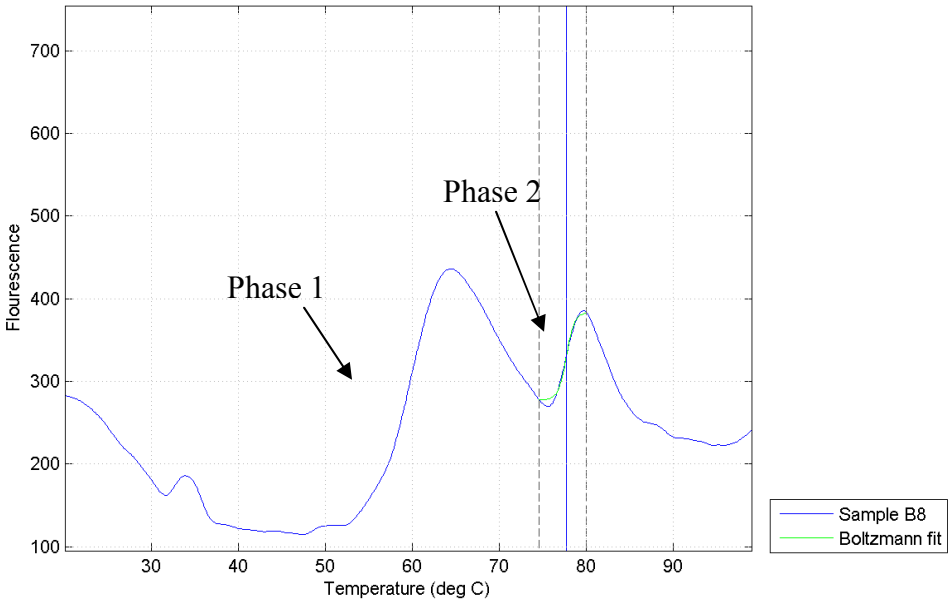

Figure 12: Multiple melt phases

Analysis tips

- **To generate plots of data from multiple wells:** The Tmtool™ application provides plot of data from a single well. To generate plots of data from multiple wells together, use the data output in example_SmoothedmeltCurves.txt produced when the "Export Plot Data" option is selected prior to the analysis.

- **Review data plots**: It is recommended that, for quality purposes, all Tm values generated by the tool be reviewed by observing the data plots.
  - For example, for well B9, there is a low signal flag raised. Well B9 data is presented in Figure 13: Well with warning flag "Low Signal". The signal was very low, compared to the highest magnitude of the signal seen in that group of wells analyzed together. This result should be rejected.

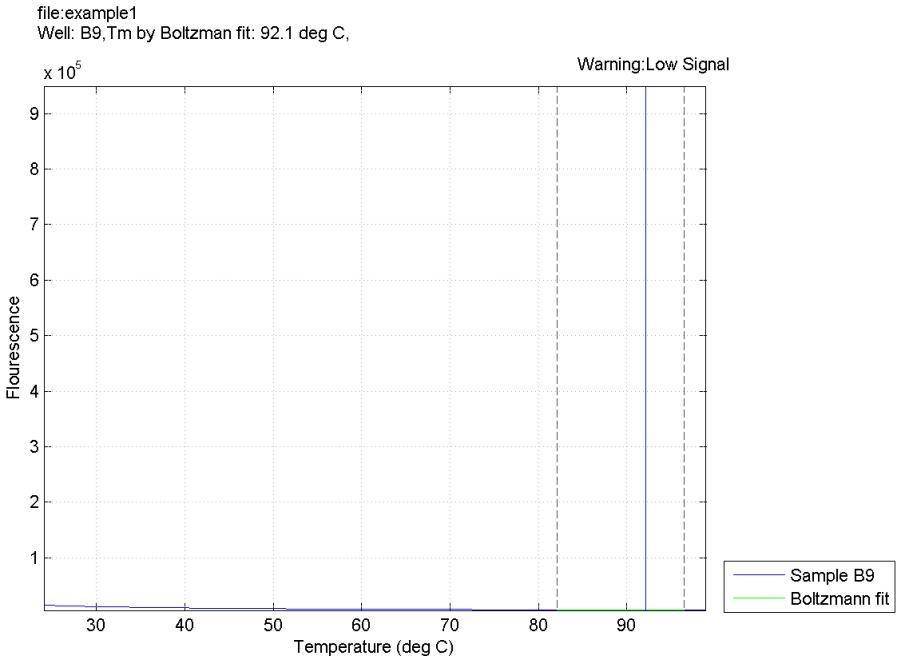

Figure 13: Well with warning flag "Low Signal"

- Another example is presented in Figure 14. The automated region of selection in this case was too broad which resulted in a poor fitting to the data. Fitting can be improved by a good choice of the region of analysis. Look at section 4.11 to learn how to define custom region of analysis for more accurate results.

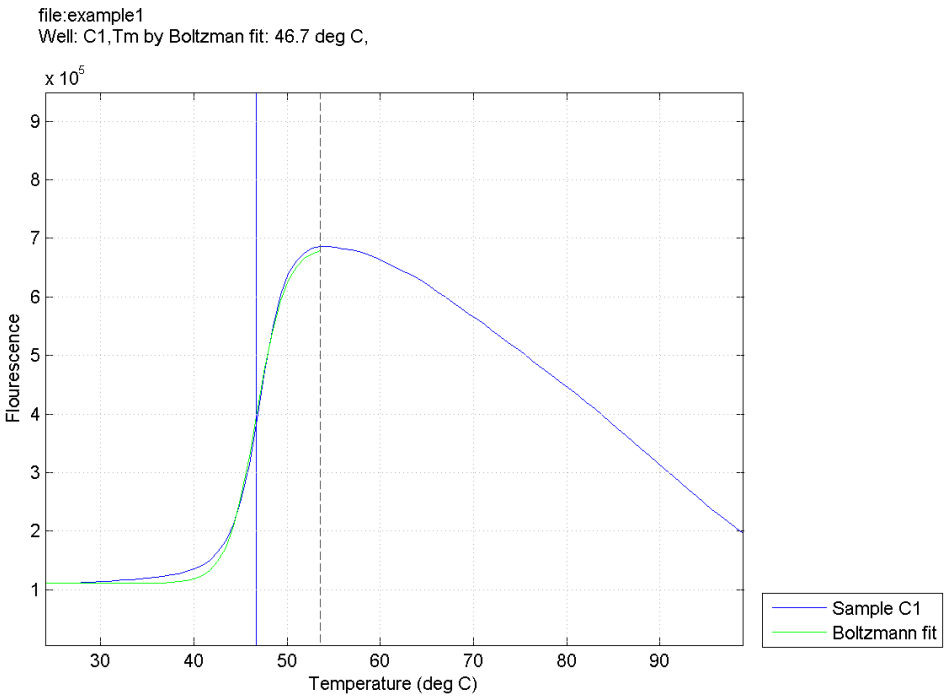

Figure 14: An example of where a custom region of analysis selection is recommended

- Flags: The software applies flags to a broad range of results. For flagged results, review the Tm value and the Boltzmann fit on the plot and if the results are reasonable, you may choose to ignore the flag.

6 Definitions

Table 1: Warning/Error Messages and their Definitions

Warning Message	Definitions
Empty Well	The well contains no data.
Custom Region	User-designated region of analysis instead of using default region of analysis auto selected by the tool.
Low Signal	The ratio of average signal value in well to the absolute maximum seen in the set of data from wells being analyzed (excluding omitted wells) is low
Few Points In Region of Analysis	The Boltzmann fitting is performed with less than 5 points because of a very short region of positive slope observed in the data.
Fitting with warning	If a good convergence was not reached in the fitting process, this warning is supplied.
Custom Region, Boltzmann failed	If a custom region of analysis was selected, but Boltzmann fitting did not execute properly, this error flag is generated.
Undetermined	Additional errors such as a fitting error or a region of analysis selection error happened inside the algorithm for which a Tm generation failed for the well.

Table 2: Acronyms/Terms and Definitions

Acronyms	Definitions
NPC	No Protein Control
LC	Ligand Control
Tm	The inflection point in a temperature series data that is ideally a sigmoid.
Reference Well, ΔTm	One or more data wells may be designated as a reference well. Each well will have its own Tm value based upon data obtained from that well. If one or more reference wells are designated, an average of the Tm values corresponding to the reference wells is calculated. ΔTm, defined as difference between average Tm value of reference wells and the Tm value of the given well will be calculated for each well.
Undetermined	If the data from a well is such that a reliable Tm cannot be generated, a Tm value of Undetermined is returned. User can force the software to return a Tm value nevertheless by selecting custom region of analysis.
Region of analysis	A region of the melt curve data is automatically selected by the software as the region of analysis. The data from the region of analysis is fit to the Boltzmann equation to return a Tm value. The algorithm looks for a region of the curve with the steepest slope increase and uses that as the region of analysis. User may choose to override this auto selected region by selecting custom region of

	analysis.
Instrument Type	The Applied Biosystem Real-time instrument on which the experiment was run needs to be selected to ensure that software applies the algorithm parameters that are suitable for the chosen instrument.

Appendix

Boltzmann Fitting Method

The Boltzmann equation: $F(T) = F(pre) + \frac{[F(post) - F(pre)]}{1 + e^{\frac{(T_m - T)}{C}}}$

where:

- F(pre) is the fluorescence reading before the transition or melting at the start of the analysis region
- F(post) is the fluorescence reading after the transition or melting, at the end of the analysis region
- T_m is the melting temperature
- C is the enthalpy of the reaction

Data are fit to this equation to generate the T_m.