# Skyline ABRF sPRG Data Processing Tutorial

This tutorial covers how to process your label-free quantitation data using the LC-MS instrument platform of your choice, upload peak integration results to Panorama, and export peak area ratios for submission to the ABRF sPRG.

In this tutorial you will work with data from a triplicate set of label-free LC-MS data.


This tutorial will cover the follow area critical to making effective use of Skyline for analysis of your ABRF sPRG study sample.
- Generating Skyline spectral libraries from discovery datasets
- Setting up a Skyline document for either MS 1 or data independent acquisition (DIA) filtering
- Importing raw data and using the retention time information for spectral libraries to direct peak picking during MS1 or DIA filtering
- Export results using a Skyline report template
- Uploading your Skyline results directly to Panorama
- Uploading your raw data and results to NIST server
- Filling out the ABRF sPRG 2013 Study Survey

 Skyline is a freely-available, vendor-neutral platform for targeted proteomics investigation. It can import raw data for MS1 and DIA filtering from the instrument vendors AB Sciex, Agilent, Bruker, Thermo-Scientific and Waters. The ability to import data across various instrument platforms greatly facilitates cross-instrument comparisons and large multi-site studies.

## Getting Started
To start this tutorial, download the following Zip file:

https://panoramaweb.org/labkey/files/ABRF%20sPRG/ABRF_sPRG_Skyline_Tutorial_Data.zip

Extract the files in it to a folder on your computer, like

 C:\Users\brendanx\Documents

This will create a new folder:

C:\Users\brendanx\Documents\ABRF_Skyline

It will contain all the files necessary for this tutorial. Now start Skyline, and you will be presented with a new empty document.

## Configuring a Skyline Document for a Data Dependent Dataset

Data was collected on the AB Sciex 5600 TripleTOF in three technical replicates with LC-MS label-free analysis (Figure 1).

Table 1. Filenames and Samples used for data analysis

| File Name (wiff) | Sample Name | MASCOT results file (dat) |
|---|---|---|
| 5600TT13-1070 | Colangelo_ABRF HEK and JPT_042413_90minIDA_1_375_1_1 | 5600TT13-1070 |
| 5600TT13-1073 | Colangelo_ABRF HEK and JPT_042413_90minIDA_1_375_1_2 | 5600TT13-1073 |
| 5600TT13-1076 | Colangelo_ABRF HEK and JPT_042413_90minIDA_1_375_1_3 | 5600TT13-1076 |
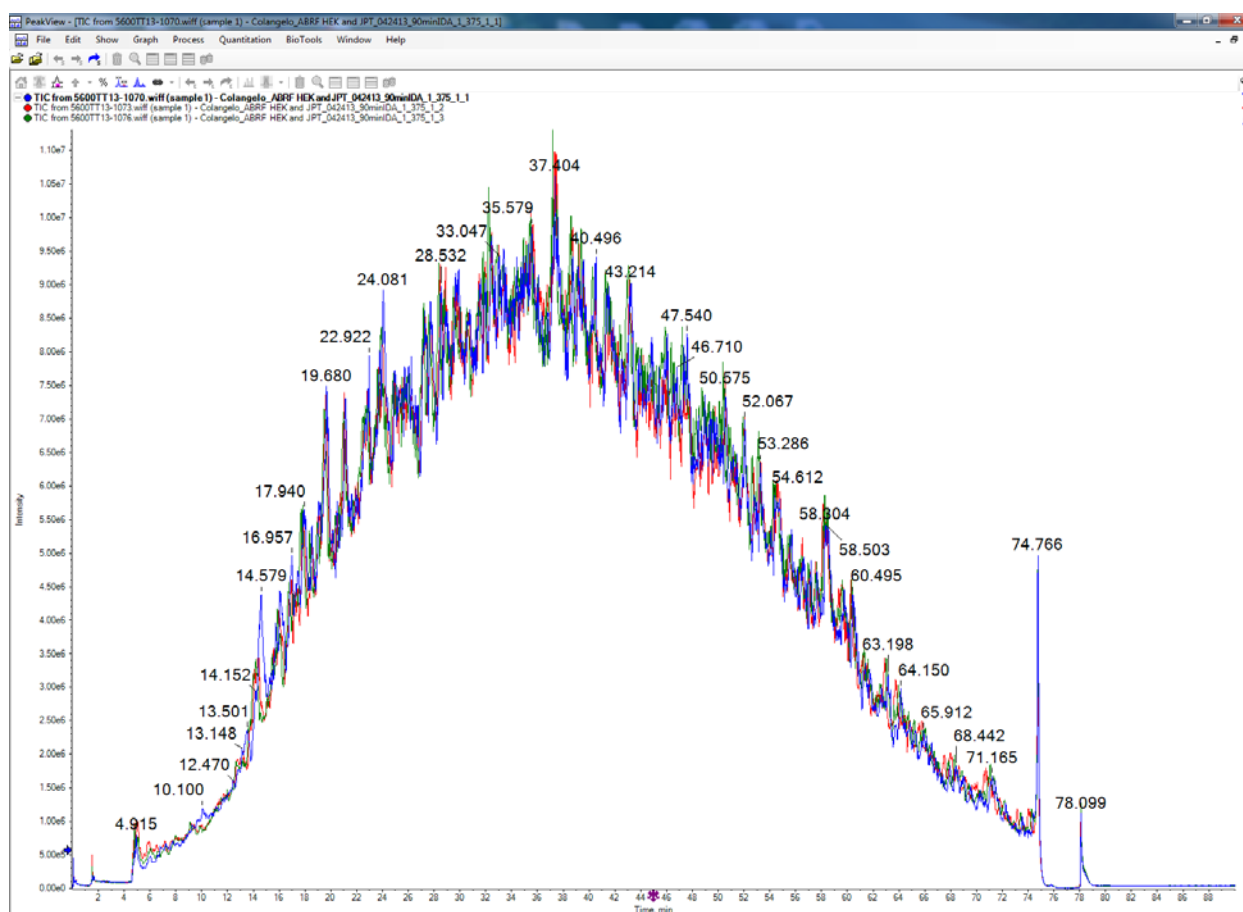


Figure 1. Total Ion Chromatogram Overlay from 90min LC-MS label-free runs on ABRF sPRG 2013 Study Samples

After data collection, the resulting LC-MS label free runs, the wiff files were converted to mgfs using the AB Sciex Data Converter (beta 1.3), and searched by MASCOT (2.4.0) using the ABRFsPRG2013_v1.fasta database and the following parameters.

*Search parameters*

| | |
|---|---|
| **Type of search** | : MS/MS Ion Search |
| **Enzyme** | : Trypsin |
| **Fixed modifications** | : ☑Carbamidomethyl (C) |
| **Variable modifications** | : ☑Deamidated (NQ), ☑Label:13C(6)15N(2) (K), ☑Label:13C(8)15N(2) (R), ☑Oxidation (M) |
| **Mass values** | : Monoisotopic |
| **Protein mass** | : Unrestricted |
| **Peptide mass tolerance** | : ± 25 ppm (# $^{13}$C = 1) |
| **Fragment mass tolerance** | : ± 0.2 Da |
| **Max missed cleavages** | : 1 |
| **Instrument type** | : ESI-QUAD-TOF |

The resulting Mascot .dat files are included in the ZIP file for this tutorial.  Follow the steps below to build a spectral library in Skyline.
**Setttings**…..**Peptide Settings**….**Library Tab**….Click on **Build**
Name the library "ABRF_sPRG_2013_Yale"
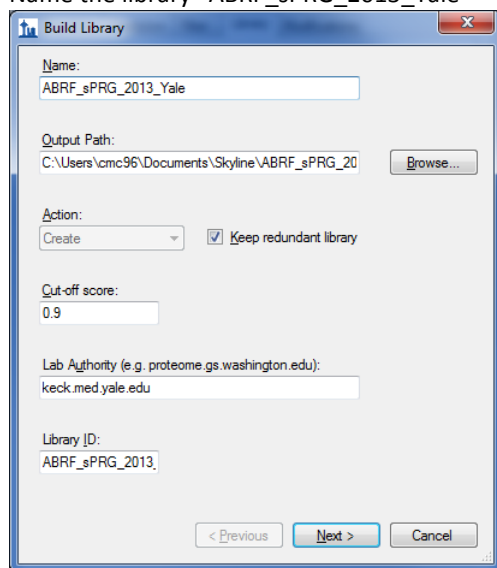


Use the **Add Files** button to add the .dat files, as shown below, from the ABRF_Skyline folder extracted from the ZIP file.
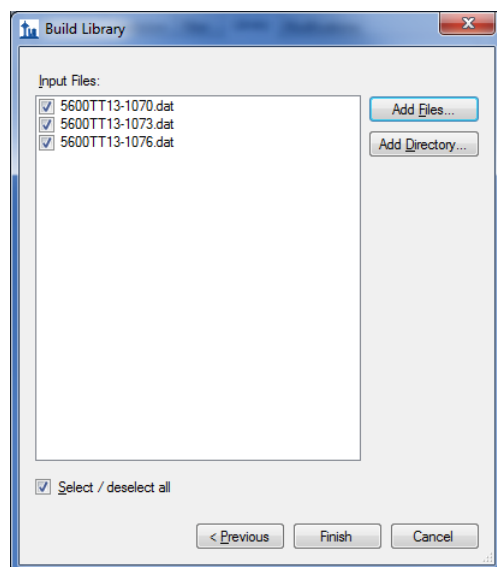
Check the checkbox for the new Spectral Library.
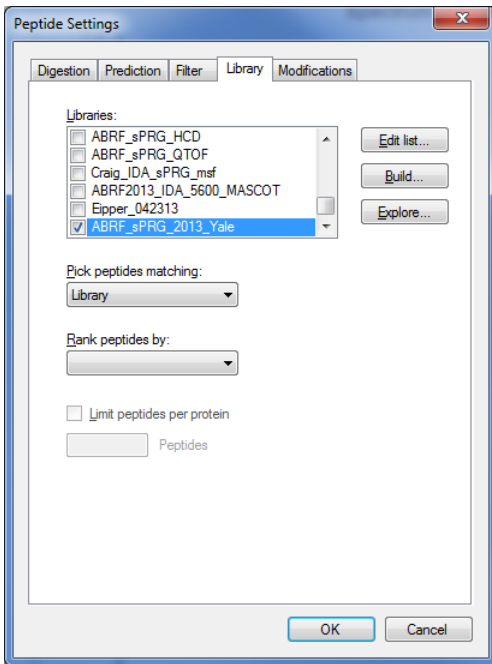


From the **Modifications** tab, check "Carbamidomethyl (C)" as a structural modification and "Label:13C(6)15N(2) (C-term K)" and "Label: 13C(6)15N(4) (C-term R)" as isotope modifications and then click **OK**.
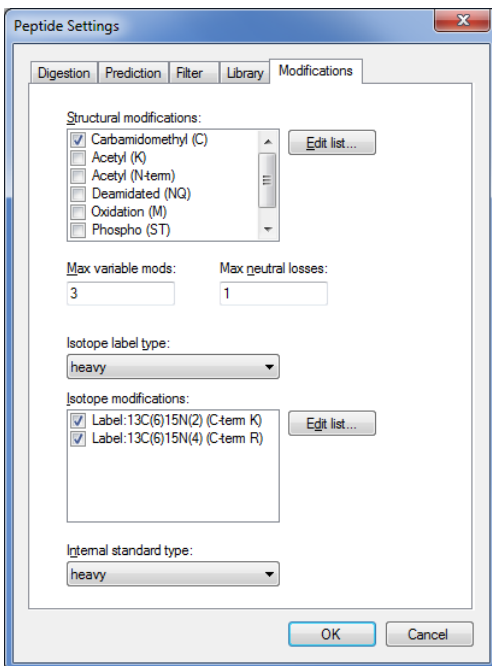
**Settings**...**Transitions settings**.....**Full-Scan** Tab

**Transition Settings**

Prediction | Filter | Library | Instrument | Full-Scan

MS1 filtering

Isotope peaks included:
Count

Precursor mass analyzer:
TOF

Peaks:
3

Resolving power:
15,000

Isotope labeling enrichment:
Default

MS/MS filtering

Acquisition method:
None

Product mass analyzer:

Isolation scheme:

Resolution:
Th

Retention time filtering

○ Include all matching scans
○ Use only scans in retention time scheduling windows
● Use only scans within  5  minutes of MS/MS IDs

OK | Cancel

**Settings**...**Transitions settings**.....**Instrument** Tab

**Transition Settings**

Prediction | Filter | Library | Instrument | Full-Scan

Min m/z:
50    Th

Max m/z:
2000    Th

☐ Dynamic min product m/z

Match tolerance m/z:
0.05    Th

Firmware transition limit:

Firmware inclusion limit:

Min time:
min

Max time:
min

OK | Cancel

**Settings**…**Transitions settings**…..**Filter** Tab…Then click **OK**.
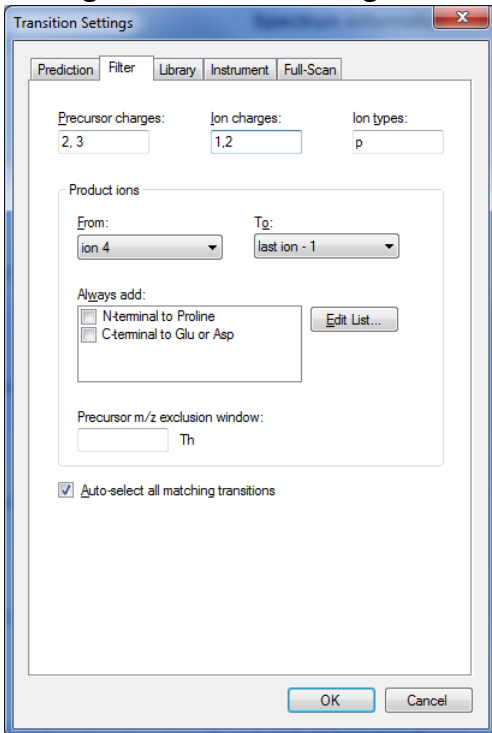
**File**….**Import**….**FASTA**…and select "ABRFsPRG2013_v1.fasta"…..click **Open**

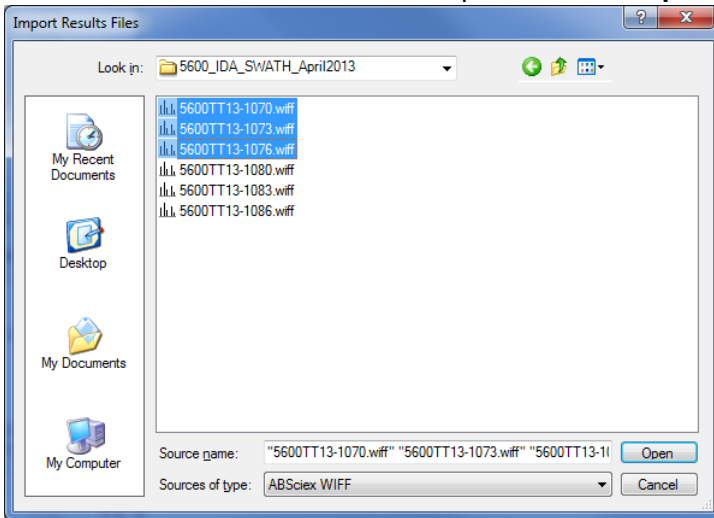A total of 975 peptide sequences were identified and imported as shown below.
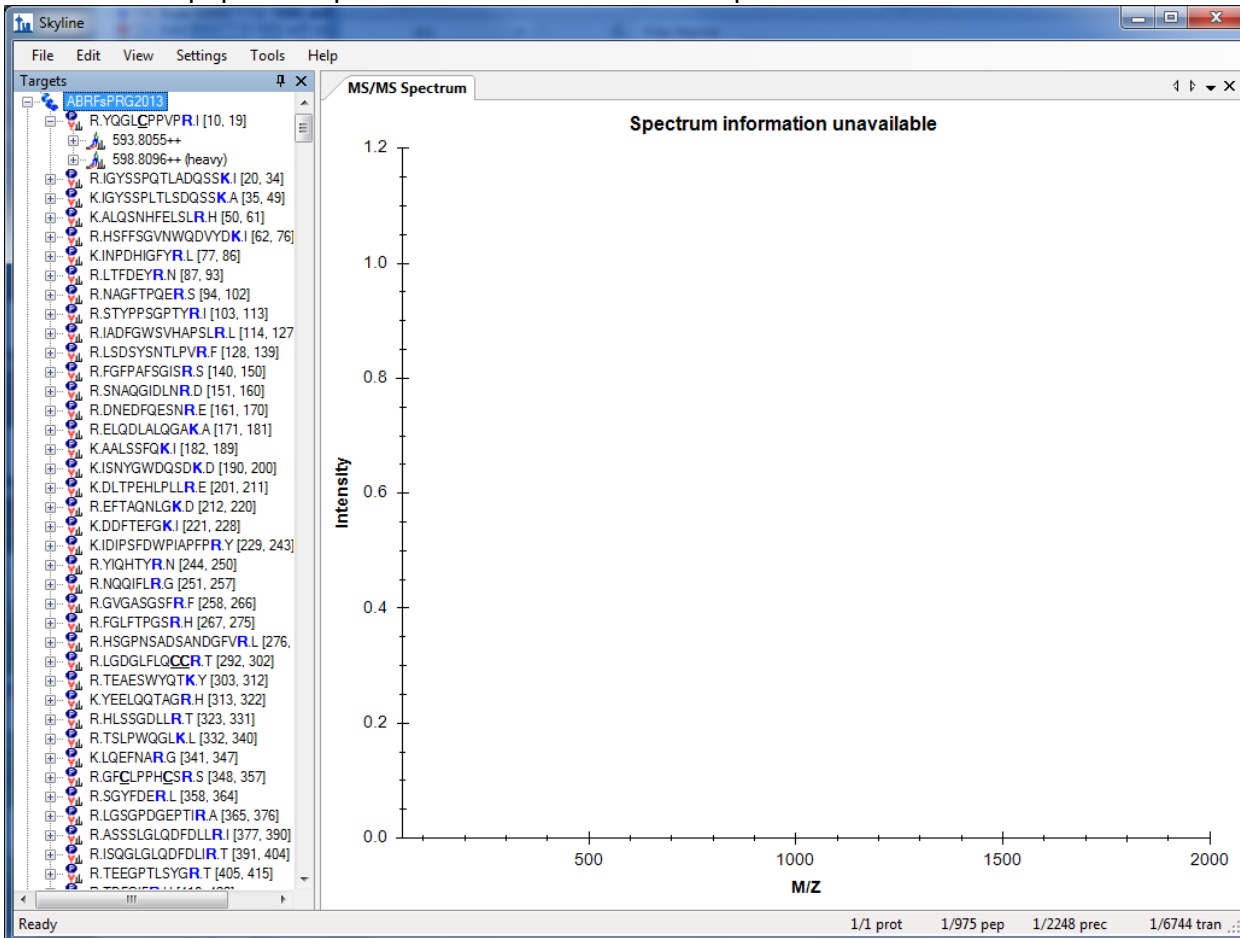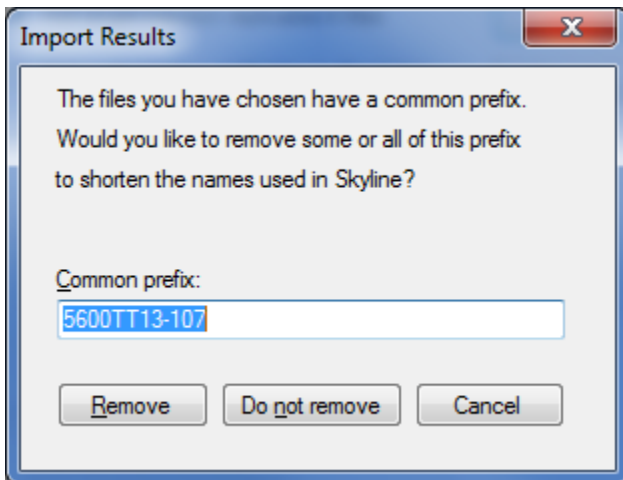
**File….Import…..Results**

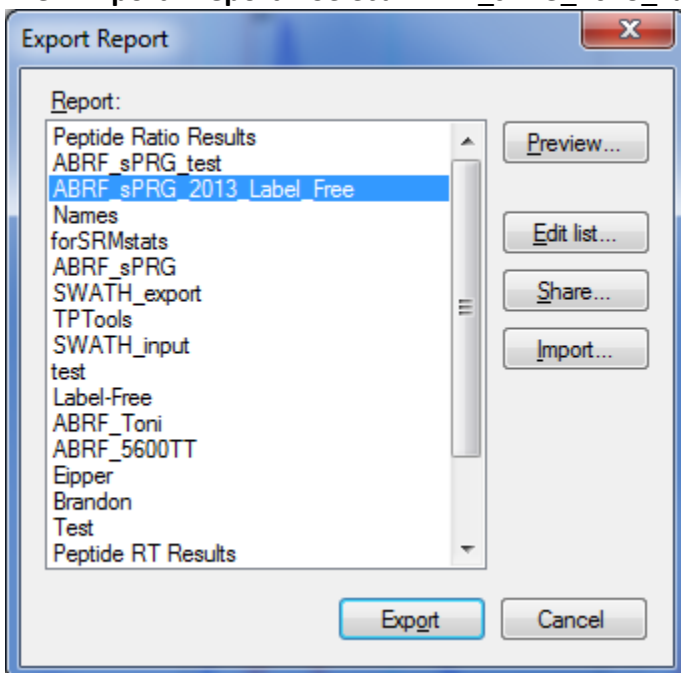Save the Results to "Yale_sPRG2013_ABRF_Label_Free.sky"**…click Save**
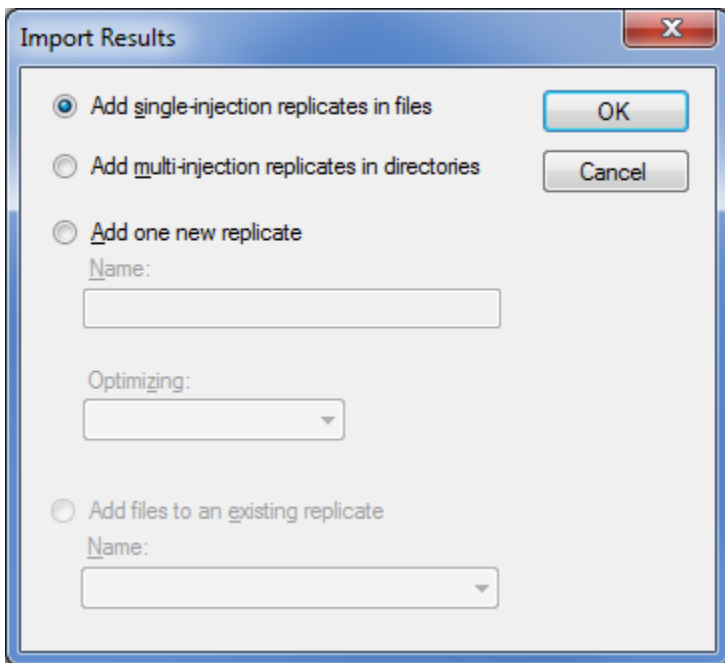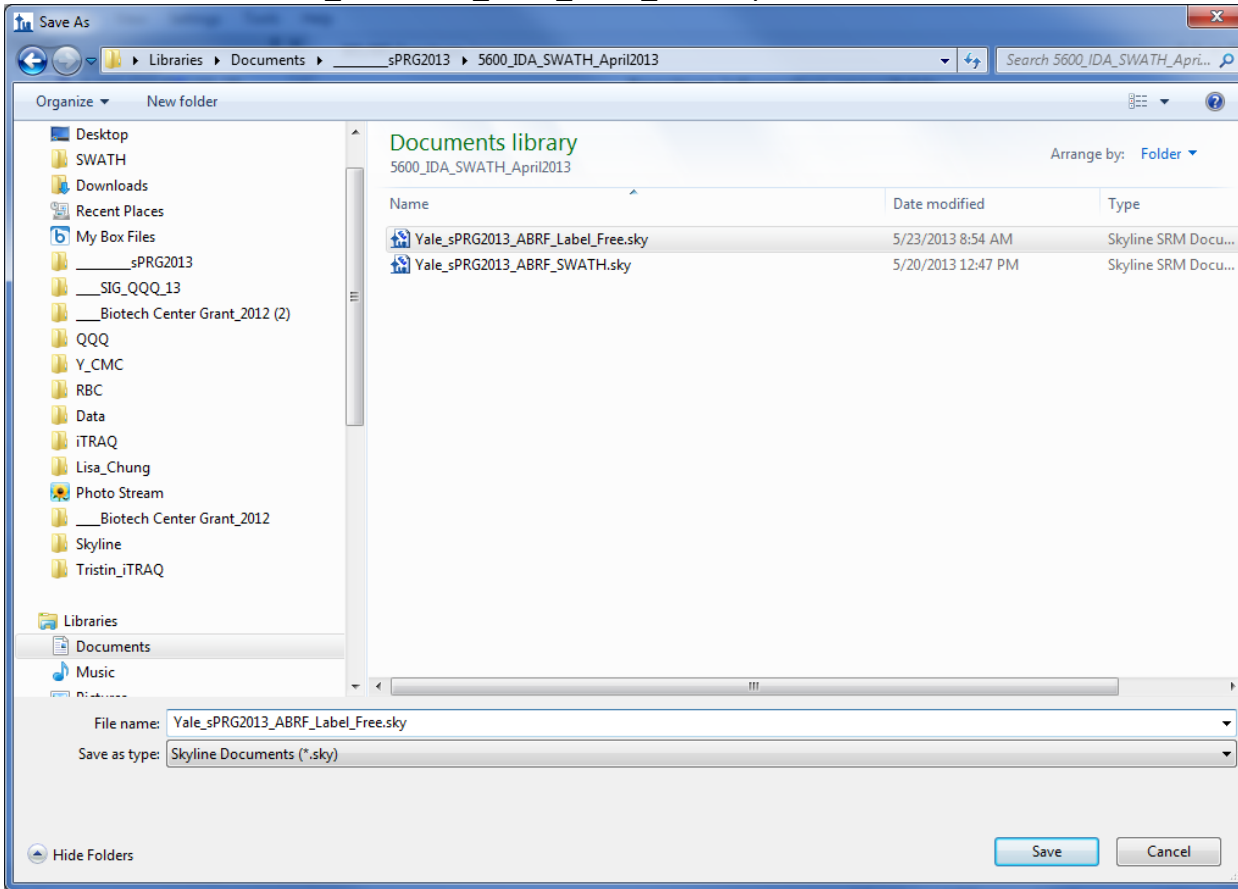
Select the three label-free wiff samples and click **Open**



Next Skyline will ask you to remove the prefix and click **Do not Remove**



**File**….**Export**…**Report**….Select "ABRF_sPRG_2013_Label_Free"



Save export "Yale_sPRG2013_ABRF_Label_Free.csv".

For your own personal data file please rename your data results file with a unique anonymous identifier composed of six alphanumeric characters (e.g. "12345A").  If you are submitting several data reports corresponding to alternative analytical strategies, keep the first six characters the same and concatenate sequential numbers (e.g., "12345A-01," "12345A-02," and "12345A-03").  Retain this identifier for future as this will be the only way for you to find your data in any presentation of our study results.  (Your data will only be referred to by its unique identifier in any presentation).
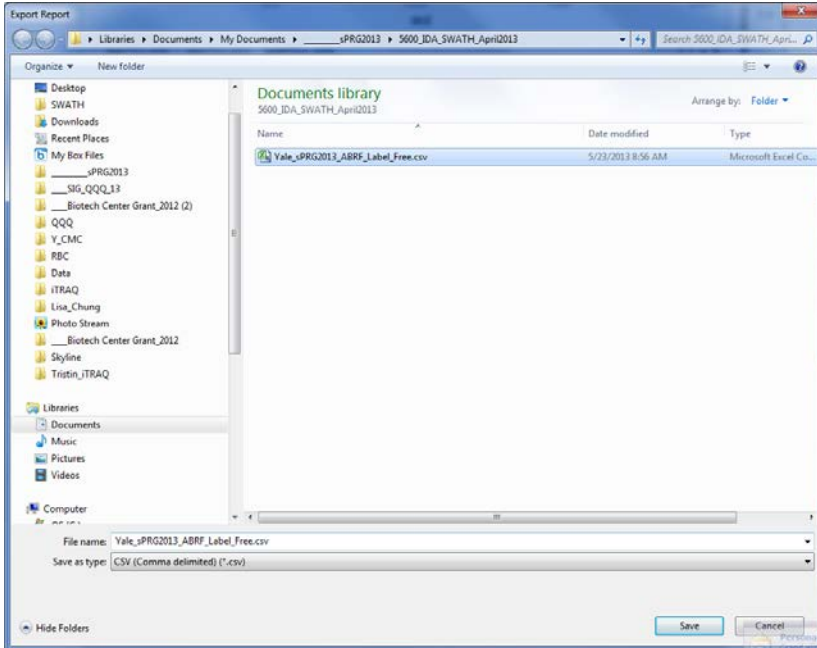


**File**…**Share**….choose complete dataset….**Save** Skyline Folder
Next you will also publish your data to Panorama……
Using Skyline go to Tools > Options and add  the PanoramaWeb.org server to Skyline.  This has to be done only once for each Panorama server that you would like to connect to from Skyline.  Use "submit" for the password.
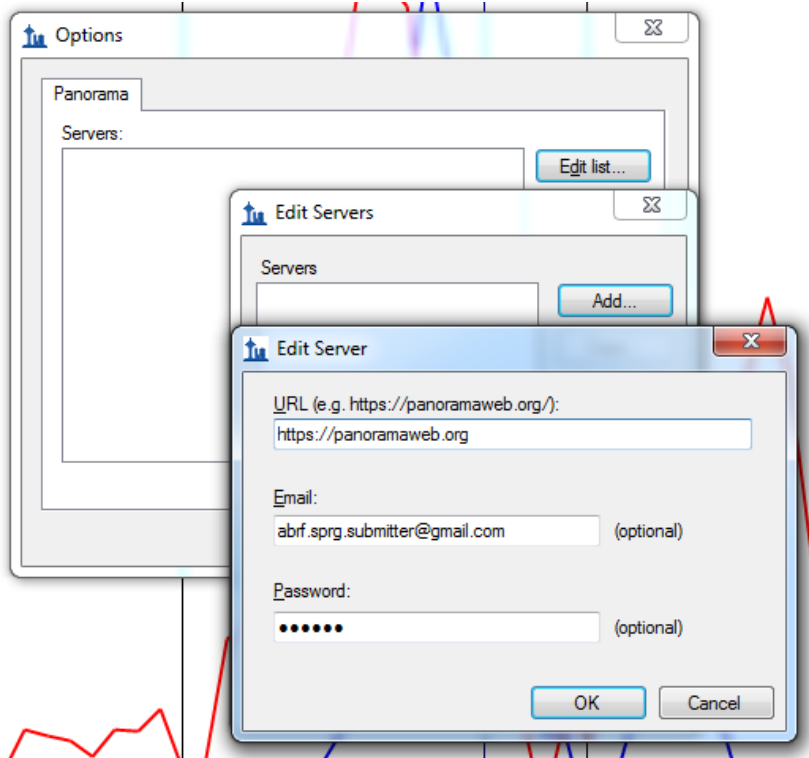
Open the Skyline document that you want to publish to a Panorama server and click on **Publish to Panorama**... in the **File** menu.  Select a folder on the server where you want to add the document.  Folders for which you do not have access will be grayed-out.
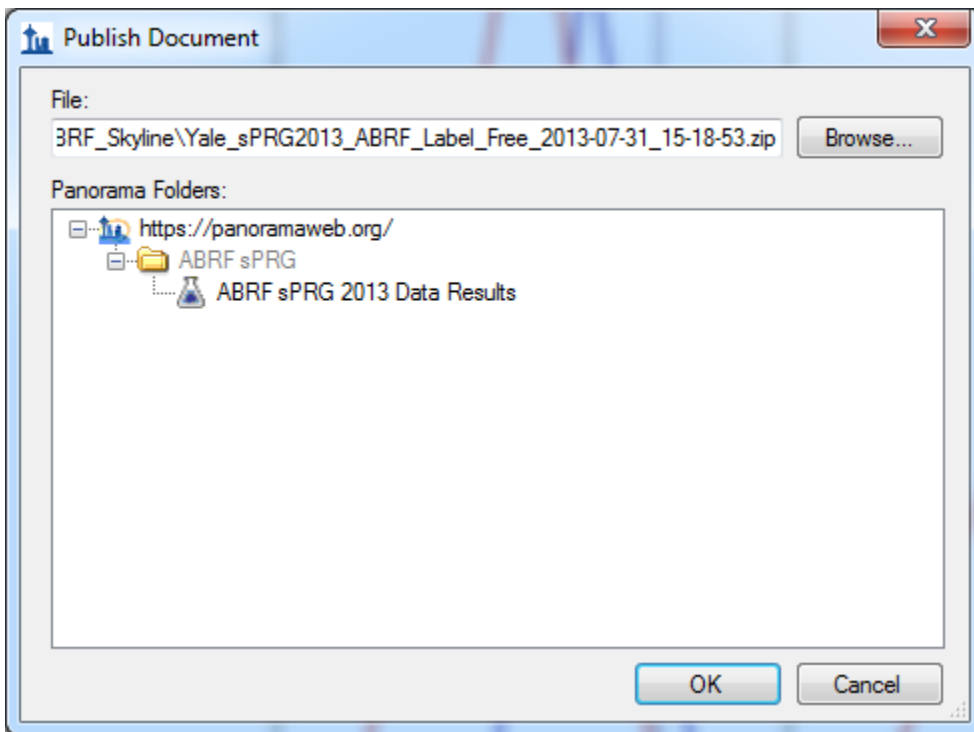


The file will be uploaded to the **server**.
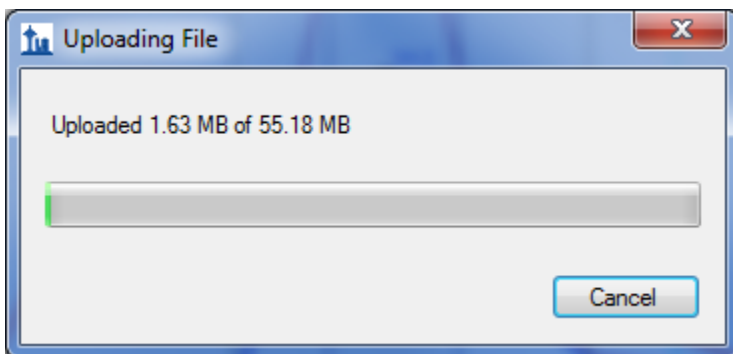
Your data should now be seen on the ABRF sPRG 2013 Panorama Results Page under the Targeted MS Runs.

https://panoramaweb.org/labkey/project/ABRF%20sPRG/ABRF%20sPRG%202013%20Data%20Results/begin.view?

If you prefer to you can upload Skyline data to the Panorama server via a web-browser
First go to Skyline and **File**…..**Share**
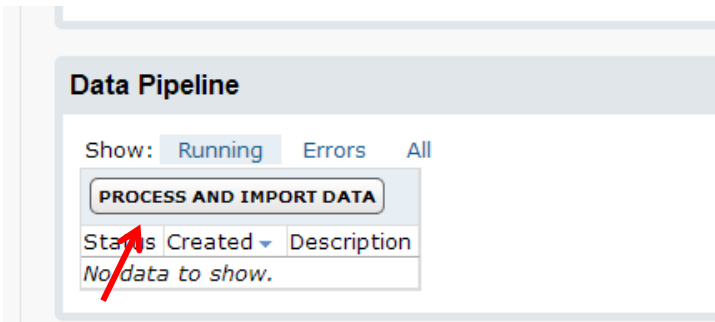You will get a pop-up box and next click the **Complete** button
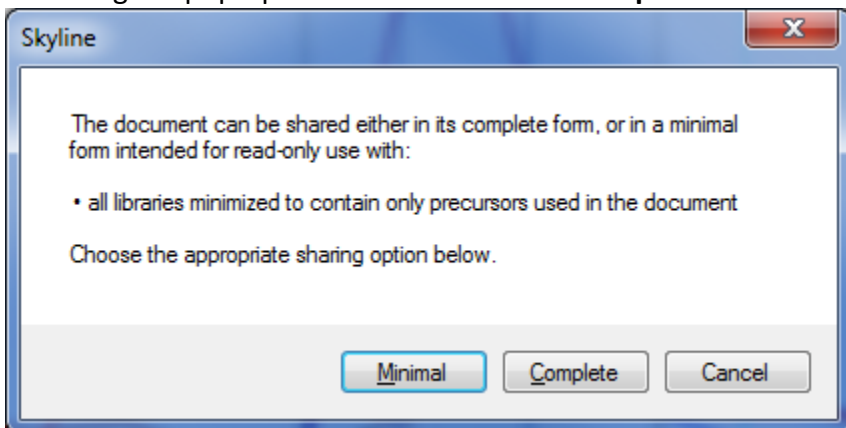


You will get a dialog box. Give your zip data results a name and click **Save**
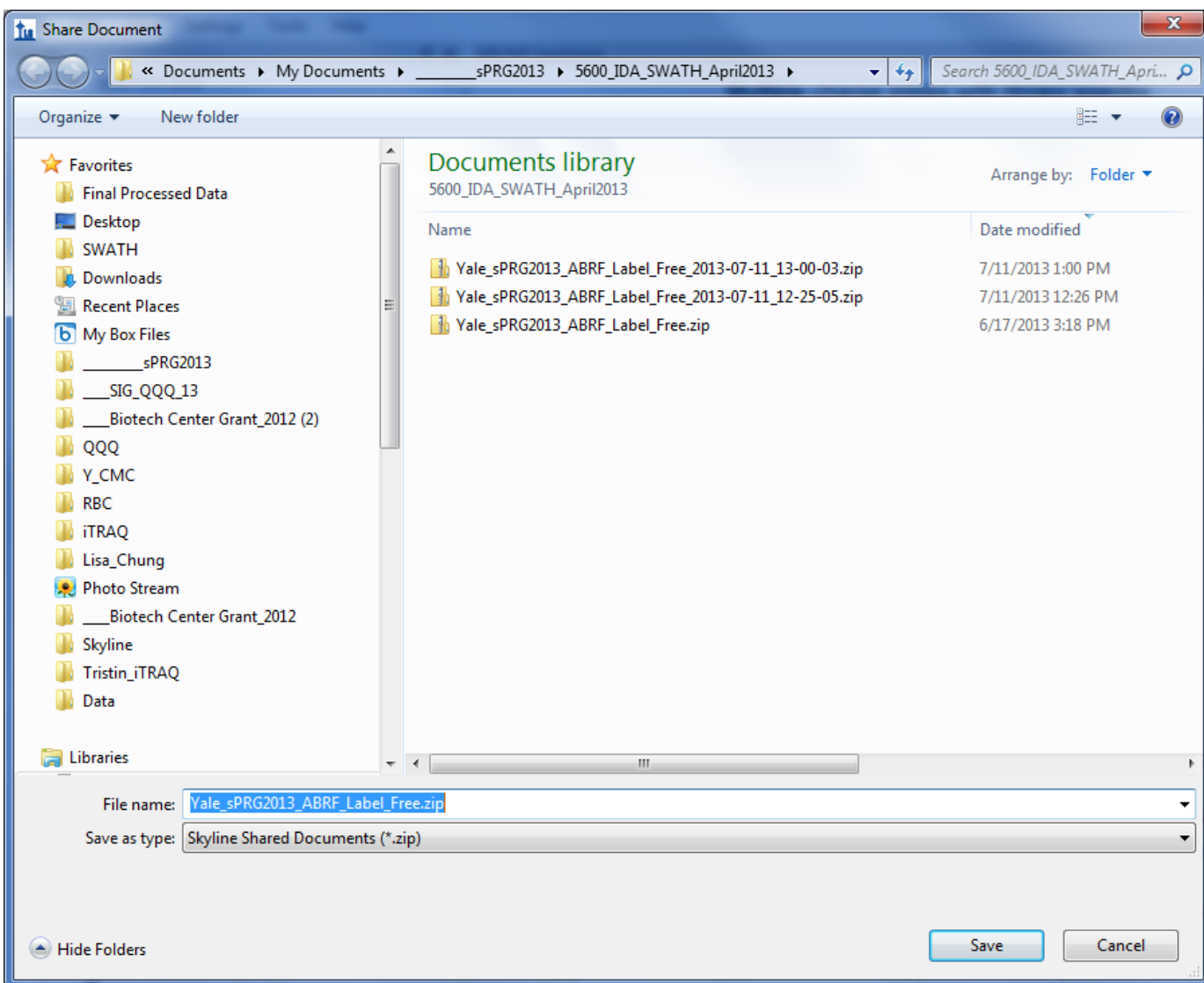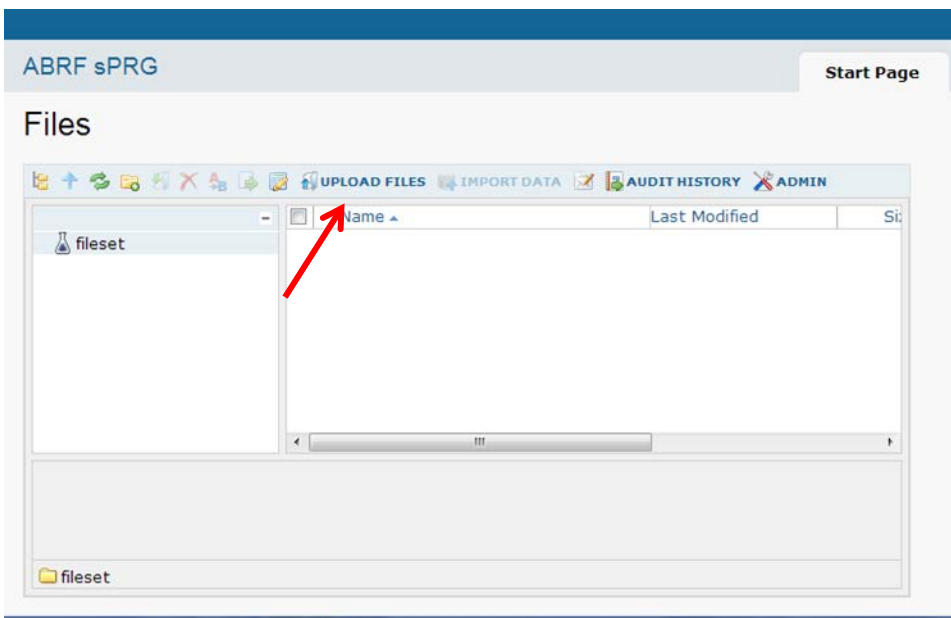
Next open a web broswer and go to
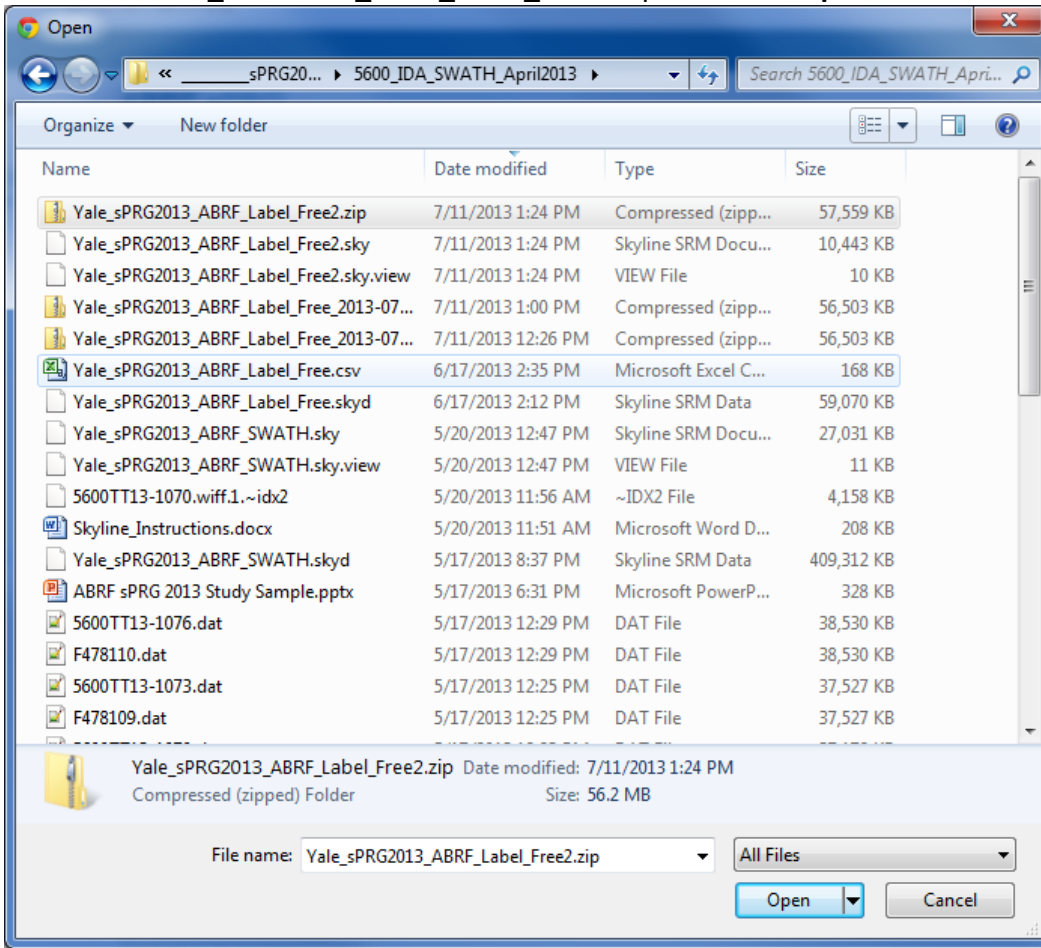https://panoramaweb.org/labkey/project/ABRF%20sPRG/ABRF%20sPRG%202013%20Data%20Results/begin.view?
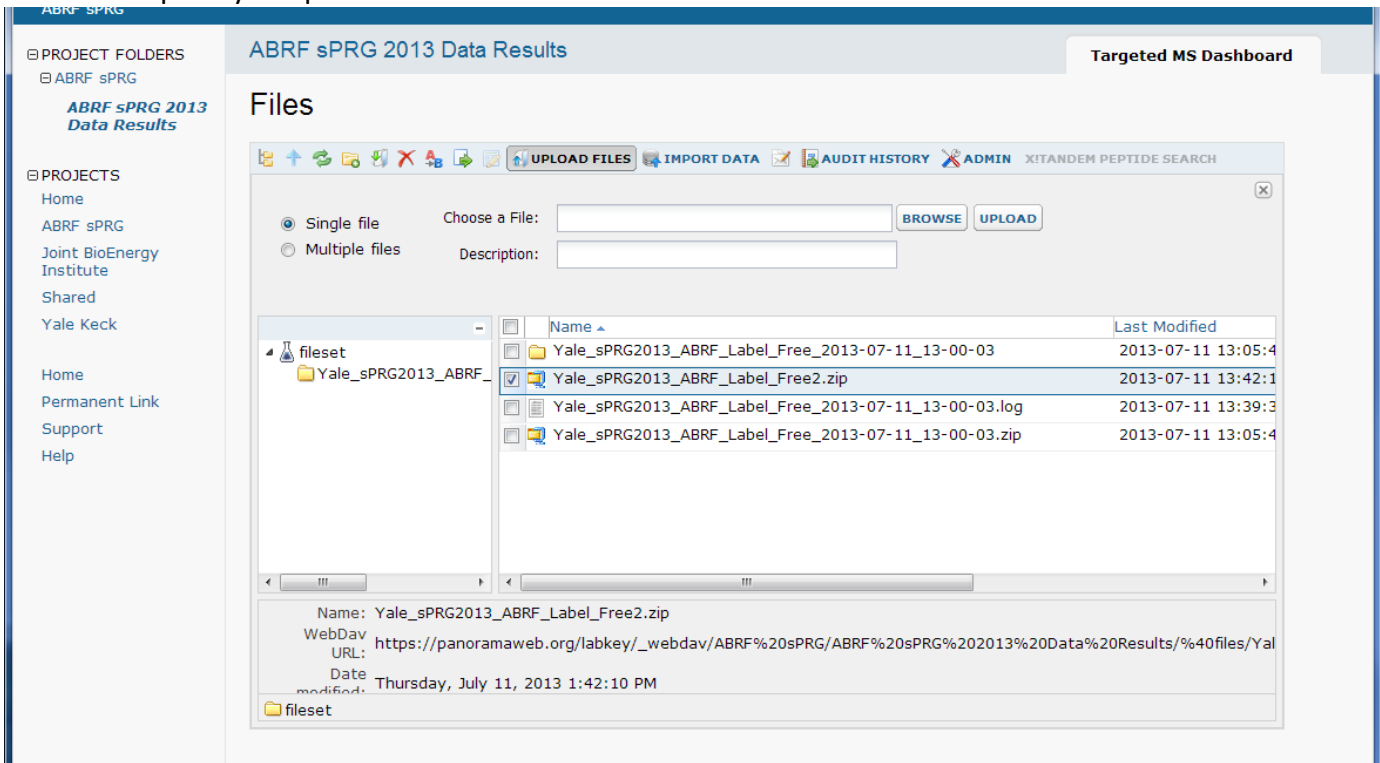At the top of the page, under Data Pipeline, click on the "PROCESS AND IMPORT DATA" link under Data Pipeline



Browse to the shared zip file that you want to import and upload it to the Panorama server.

Select the "Yale_sPRG2013_ABRF_Label_Free2.zip" and click **Open** and then Click **Upload**



Select the zip file you uploaded and click on "**IMPORT DATA**"

You will see a pop-up box asking you to import Skyline Results and click **Import**.



Finally, navigate back to the ABRF sPRG 2013 Data Results Page

https://panoramaweb.org/labkey/project/ABRF%20sPRG/ABRF%20sPRG%202013%20Data%20Results/begin.view?

Your data results will be shown under the Targeted MS Runs

## Upload RAW Data to NIST FTP Client

Use an FTP client to upload all your raw data and to ABRF sPRG 2013 ftp server

First create a folder using a unique anonymous identifier composed of six alphanumeric characters (e.g. "12345A"). Retain this identifier for future as this will be the only way for you to find your data in any presentation of our study results. (Your data will only be referred to by its unique identifier in any presentation).
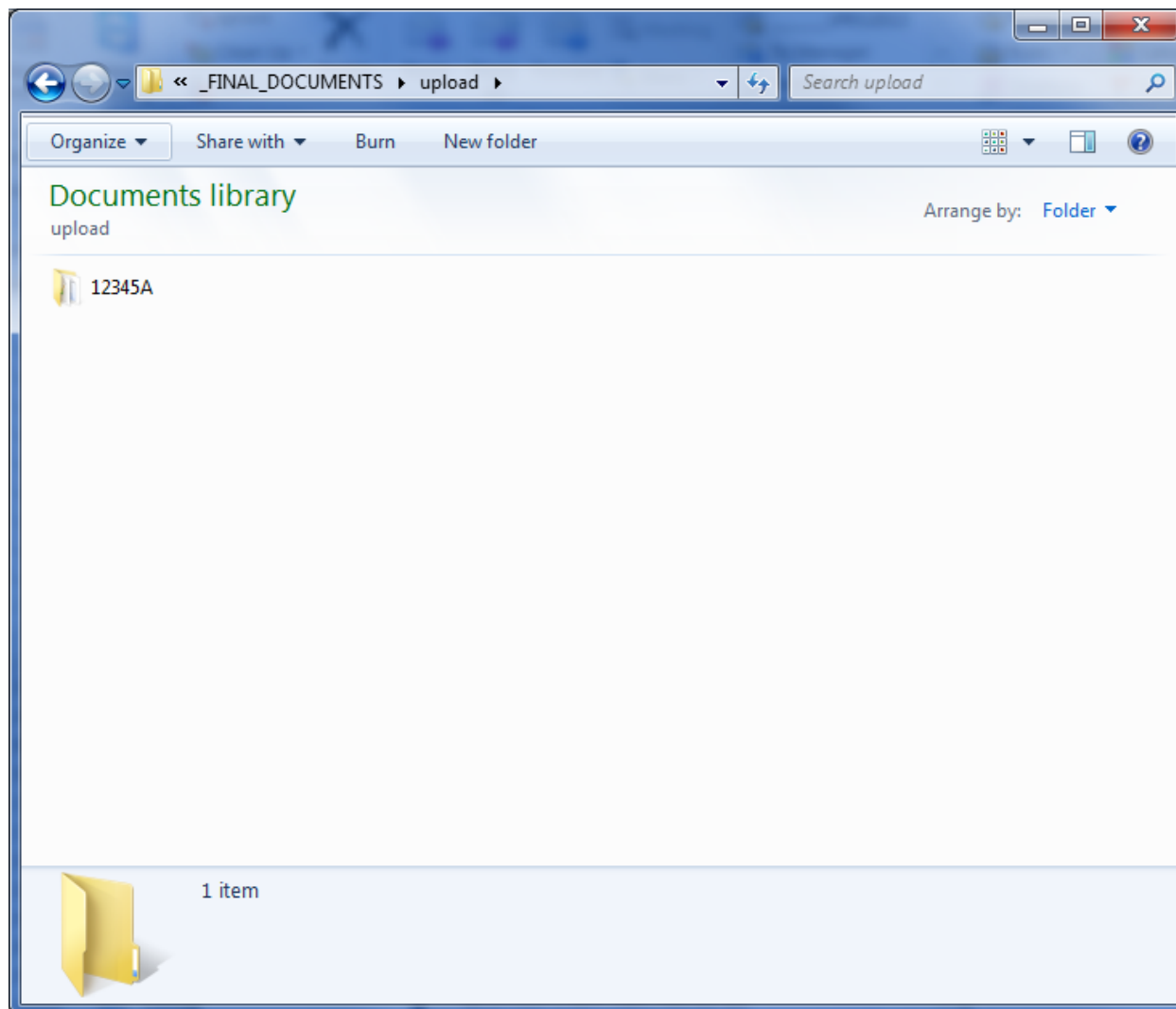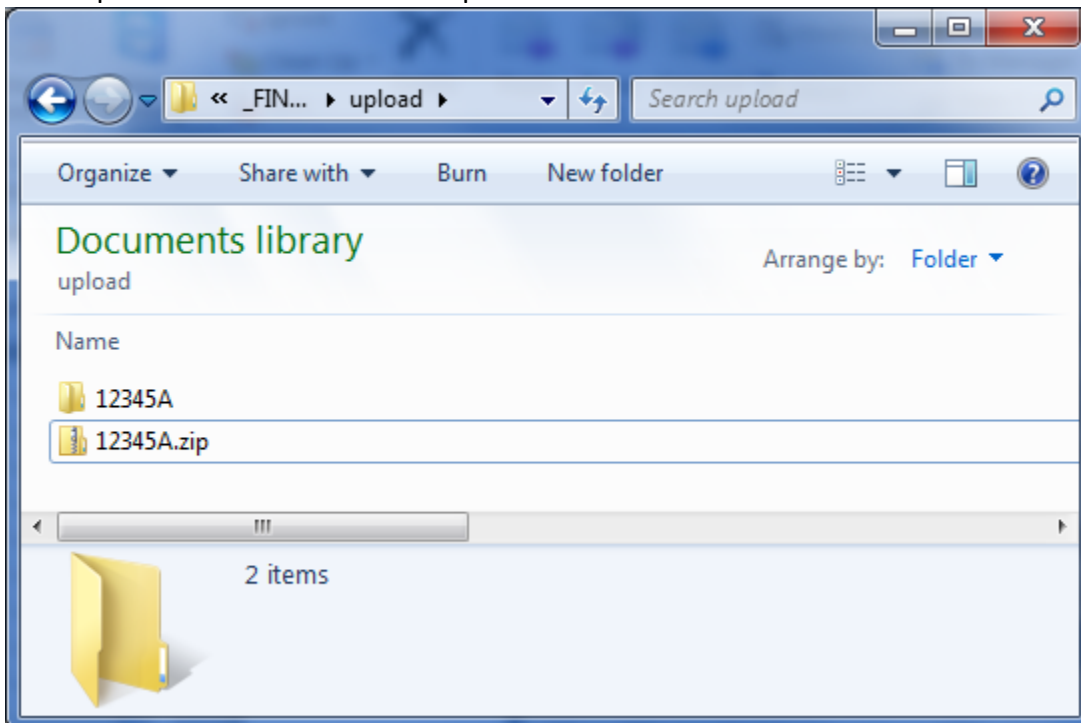
Within the folder place your Raw data files and Excel reporting template
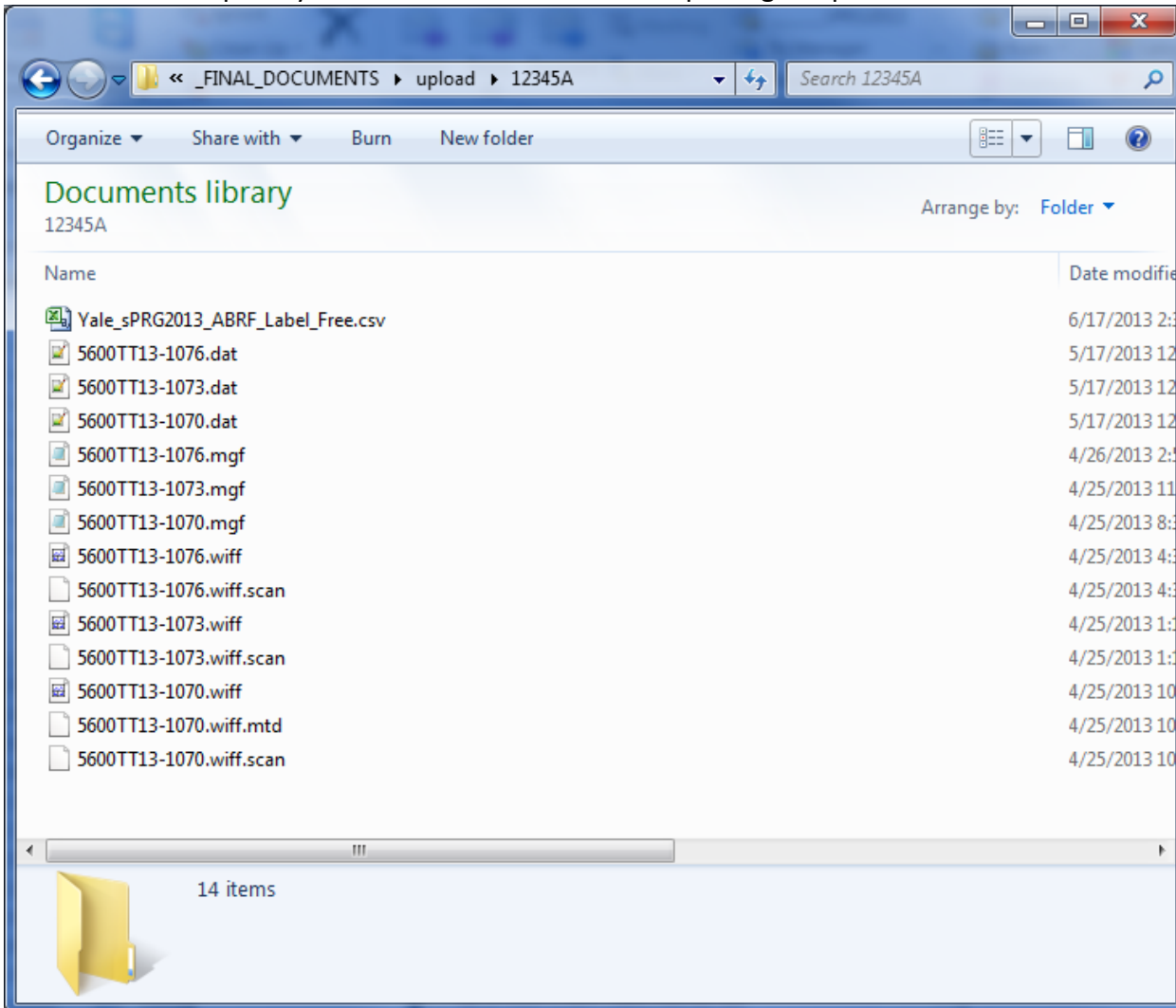


Then zip the folder with "12345A.zip

Next login to ABRF sPRG 2013 FTP server using the following login information:

<u>sPRG 2013 Participants</u>
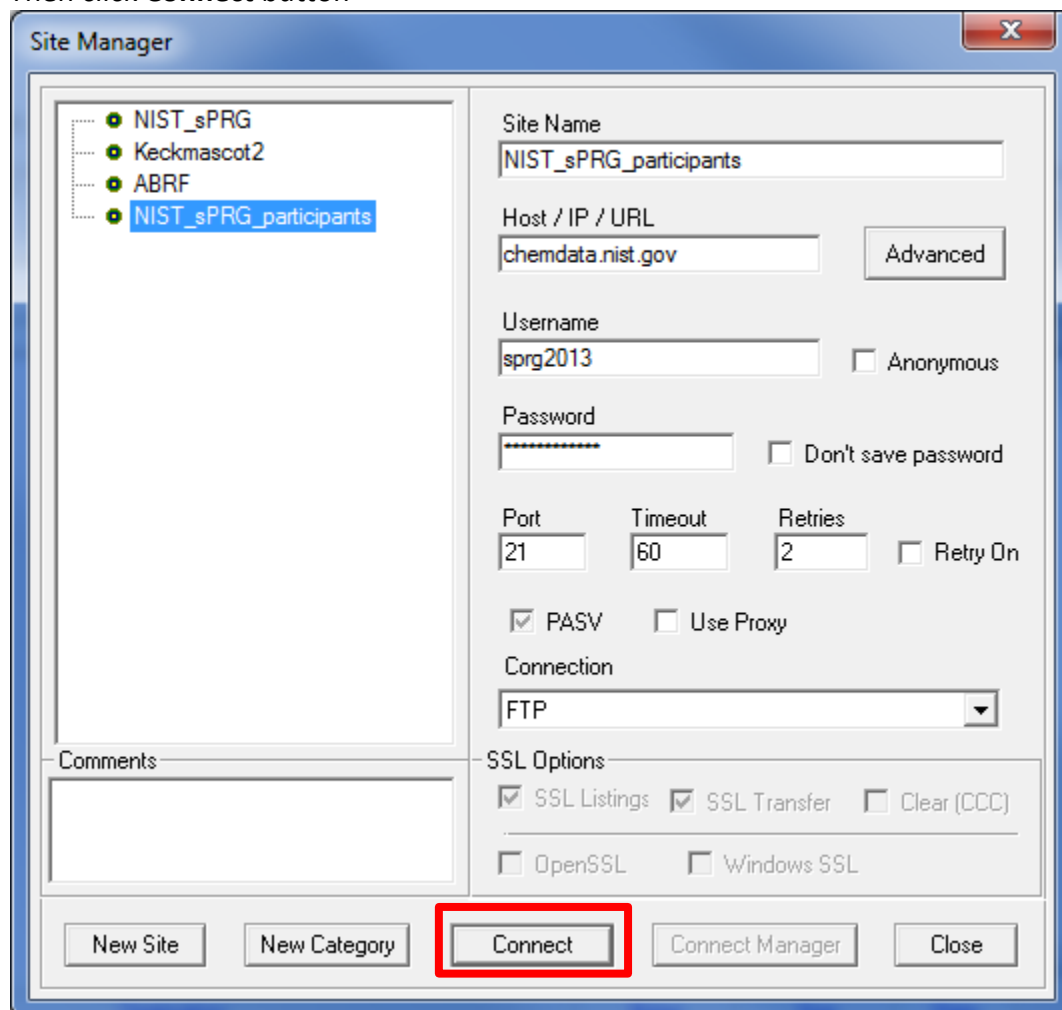Server: chemdata.nist.gov
User: sprg2013
Passwd: 2013SPRG_100

Here we are using an FTP client like CoreFTP
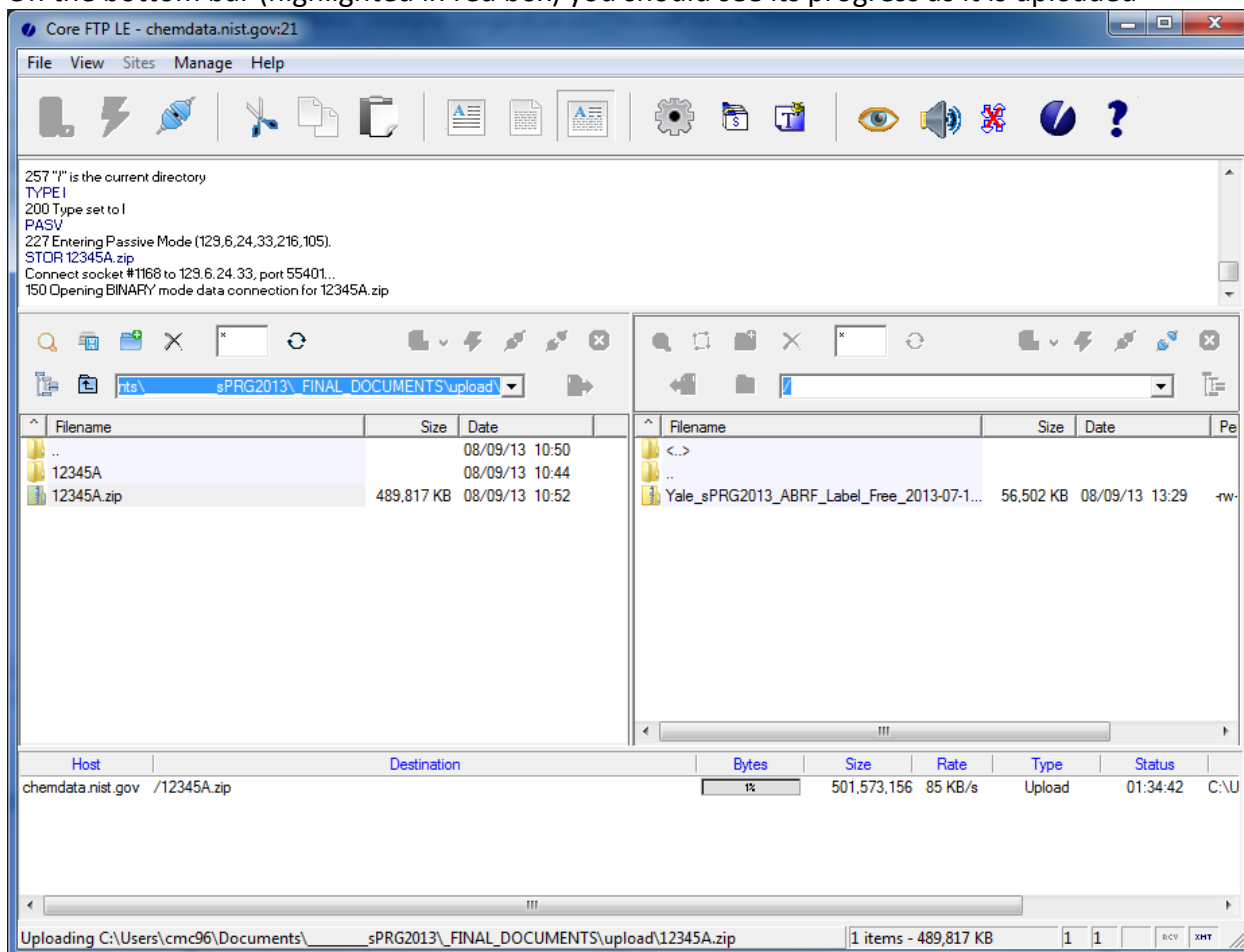Fill in the **HOST/IP/URL** with …..**chemdata.nist.gov**
Username: **sprg2013**
Password: **2013SPRG_100**
Then click **Connect** button

Drag and Drop the "12345A.zip" file to the right
On the bottom bar (highlighted in red box) you should see its progress as it is uploaded



After upload, your file should now be listed on the NIST FTP site (right hand side)

Once your data has been uploaded all that is left to do is fill out the ABRF sPRG Survey Using your web browser navigate to http://www.surveymonkey.com/s/XLMP2WV and complete the survey.