AGECalibratoR: User manual

Input data preparation

1. Run SDD-AGE as described elsewhere [Kryndushkin *et al.*, 2003] but include one or more lanes with any commercial 1 kb DNA ladder (about 2 ug is enough) mixed with loading buffer [Halfmann & Lindquist, 2009].

   Important things to remember:

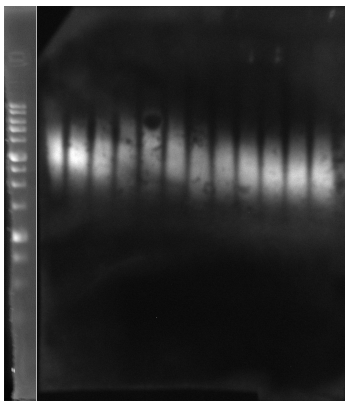
   - Record agarose concentration in the gel, running time, and voltage.
   - Do not change the voltage during the run.

2. Cut off the lane(s) with DNA ladder with any metal or plastic object sharp enough (a plastic ruler, a knife of a scalpel).

3. Stain the ladder part of the gel with ethidium bromide (e.g., 1 hour in 0.1 mg/L EtBr solution in water) or any alternative method you prefer. Please note that according to our experience incubation in EtBr should be increased about 5 times from that used for usual DNA-AGE. Take a picture of the gel. Even if you forget to cut off the ladder band before capillary transfer, you can still stain the DNA, even thought the bands might be more diffuse.

4. Use the remaining (protein) part of the gel for capillary transfer to a PVDF/nitrocellulose membrane as described elsewhere [Halfmann & Lindquist, 2009]. Carefully align either the top or the bottom of the membrane to the top/bottom of the gel.

5. Hybridize the membrane with corresponding antibody and detect the signal.



6. Combine images of the DNA ladder in the gel and western blot using gel/membrane edges for alignment with your favorite image manipulation software (e.g. GIMP or PhotoShop). If necessary adjust the images to make them white-on-black (invert colors if necessary) in 8-bit mode. Export in png or tif(f) format. An example combined image is shown at the left. Please note that you can automatically stretch contrast for the ladder lane to make the bands more clearly visible, but it is not recommended for the main image, as the weight distribution may become shifted.

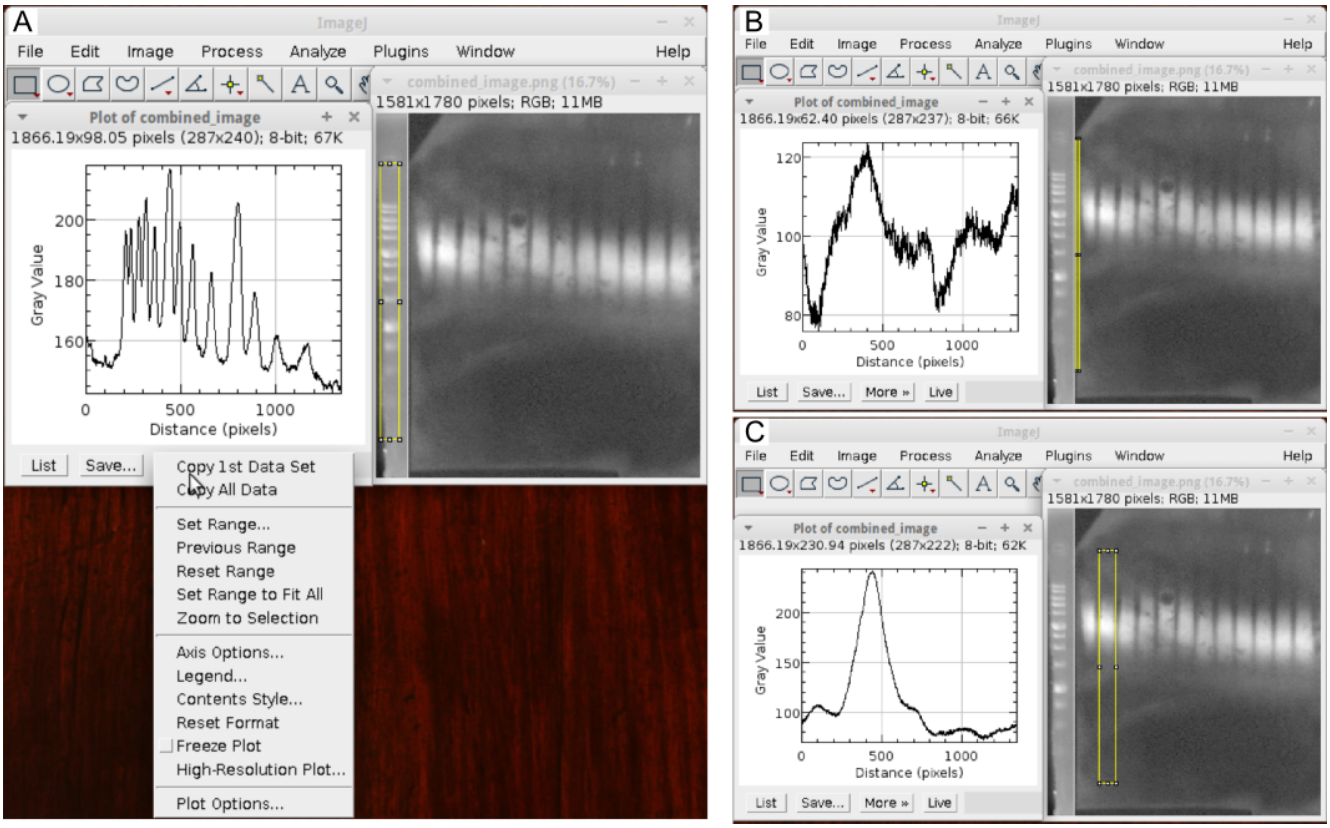
7. Load the picture into ImageJ v≥1.50

8. Collect OD profiles for all lines with ImageJ including the DNA ladder, protein samples and corresponding backgrounds.

   - Select your first lane with a rectangle. **It is necessary** to align the top edge of selection and the center of the well; otherwise, the regression model will not be correct. It is possible to change width of the selection, but not its height or vertical positioning during subsequent measurements.
   - Press Ctrl+Alt+K to get a plot profile.
   - Copy-paste data from pop-up list ('List button') into the table which will contain all profiles for the analyzed image.

- Move the selection horizontally with the arrow keys and collect the other OD profiles (repeat two previous steps).
- Do not forget to record background for protein samples. Remember that you can decrease selection width if you do not have an empty lane.

The screen shots below show example plot profiles for DNA ladder (A), background (B) and one of the sample lanes (C).



9. At this step you should have a table with the following columns: X values (e.g., `X`), background values (e.g., `background`), and values for each lane (e.g., `Sample_1`, `Sample_2` *etc.*, whatever is meaningful for you). Export as file with **comma, semicolon or tab-delimited values** (csv, csv2 or tsv) but not as a spreadsheet. Please note that the LibreOffice software has less problems saving this file than the Microsoft Office suite.

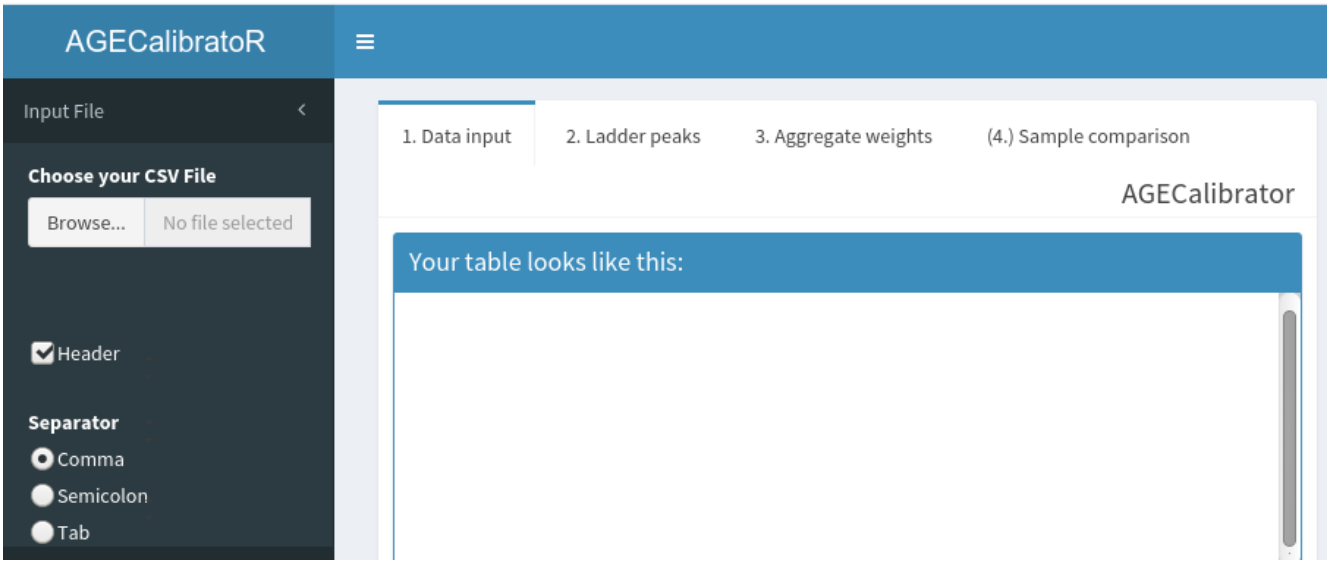
   Below is the beginning of our example table.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1	X	ladder	OT55_01	OT55_02	OT55_03	OT55_04	OT55_05	OT55_06	OT56_01	OT56_02	OT56_03	OT56_04	OT56_05	OT56_06	background
2	0	161.7619	96.179	90.333	88	87.048	90.881	92.036	90.607	90.393	97.107	100.738	98.595	110.833	94.4667
3	1	161.6786	95.357	89.488	86.929	86.095	92.131	90.857	92.929	89.476	95.536	100.905	99.69	110.024	94.6
4	2	161.7262	94.036	91.19	86.488	85.643	90.417	91.464	92.369	89.167	96.881	99.702	98.119	109.988	92.9
5	3	161.6548	93.893	91.286	87.56	86.464	91.095	90.631	92.774	88.06	97.012	100.083	99.44	111.988	93.9333
6	4	161.369	93.369	89.774	87.69	84.536	90.214	89.988	91.869	89.036	95.56	98.881	99.512	110.369	94.9667
7	5	161.0476	93.786	90.202	87.19	85.202	89.976	91.595	91.167	89.512	93.238	98.333	99.155	110.131	95.8333
8	6	160.6786	93.345	89.274	87.714	85.429	91.155	90.274	90.202	88.857	92.905	98.345	99.107	110.679	95.3
9	7	160.2143	93.321	92.429	87.833	85	92.31	90.274	90.071	88.762	93.417	98.821	99.274	109.655	92.8333
10	8	159.5833	94.762	92.31	87.357	85.167	90.619	90.44	90.655	89.81	93.643	98.107	100.31	108.702	92.9333

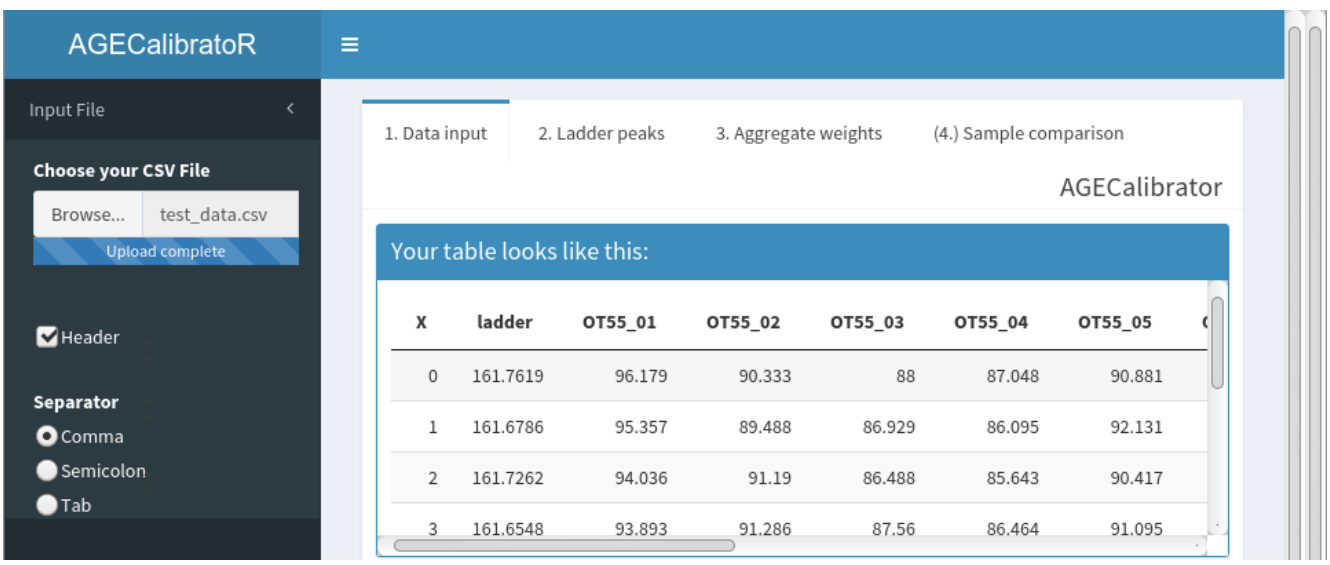
Running AGECalibratoR

1. Open RStudio, load `main.R` and press the `Run App` button (it might be more convenient to open the app in browser). Alternativerly, navigate to ...

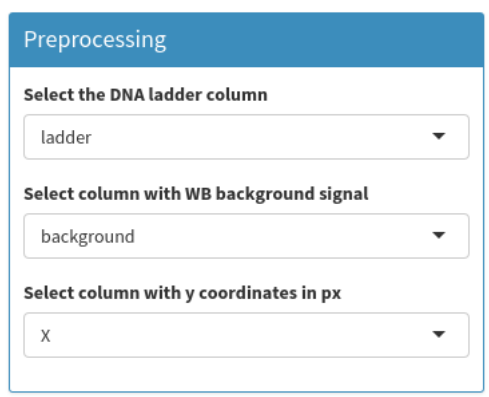
   The window should look similar to the image below.

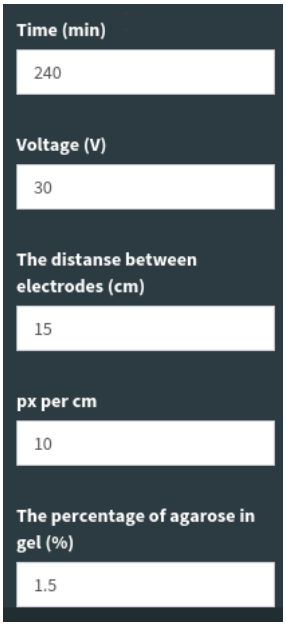


2. Select your table with the `Browse` button, choose the appropriate field separator if necessary and make sure everything worked well.



3. Set up names of columns with X coordinate, background and DNA ladder. They should be already chosen if you strictly followed the procedure above. If your column names are different from the recommended ones, this can be adjusted now: select the values from lists.





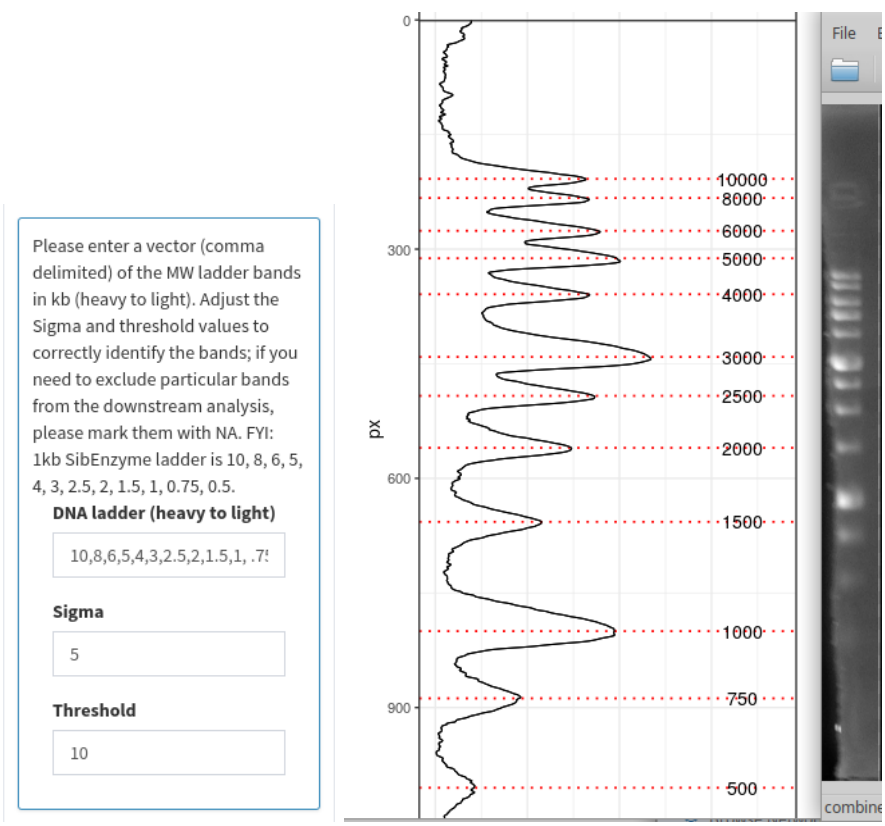
4. Now we need to specify the parameters of your experiment. It is very important for the regression. So, please specify:

   - running time in minutes;
   - voltage;
   - distance between electrode in cm;
   - the scale factor of how many pixels are in 1 cm of your image (depends on the camera or scanner you used to obtain the digital representation of the DNA ladder). The easiest way to obtain this value is to take a picture of a ruler with the same instrument.

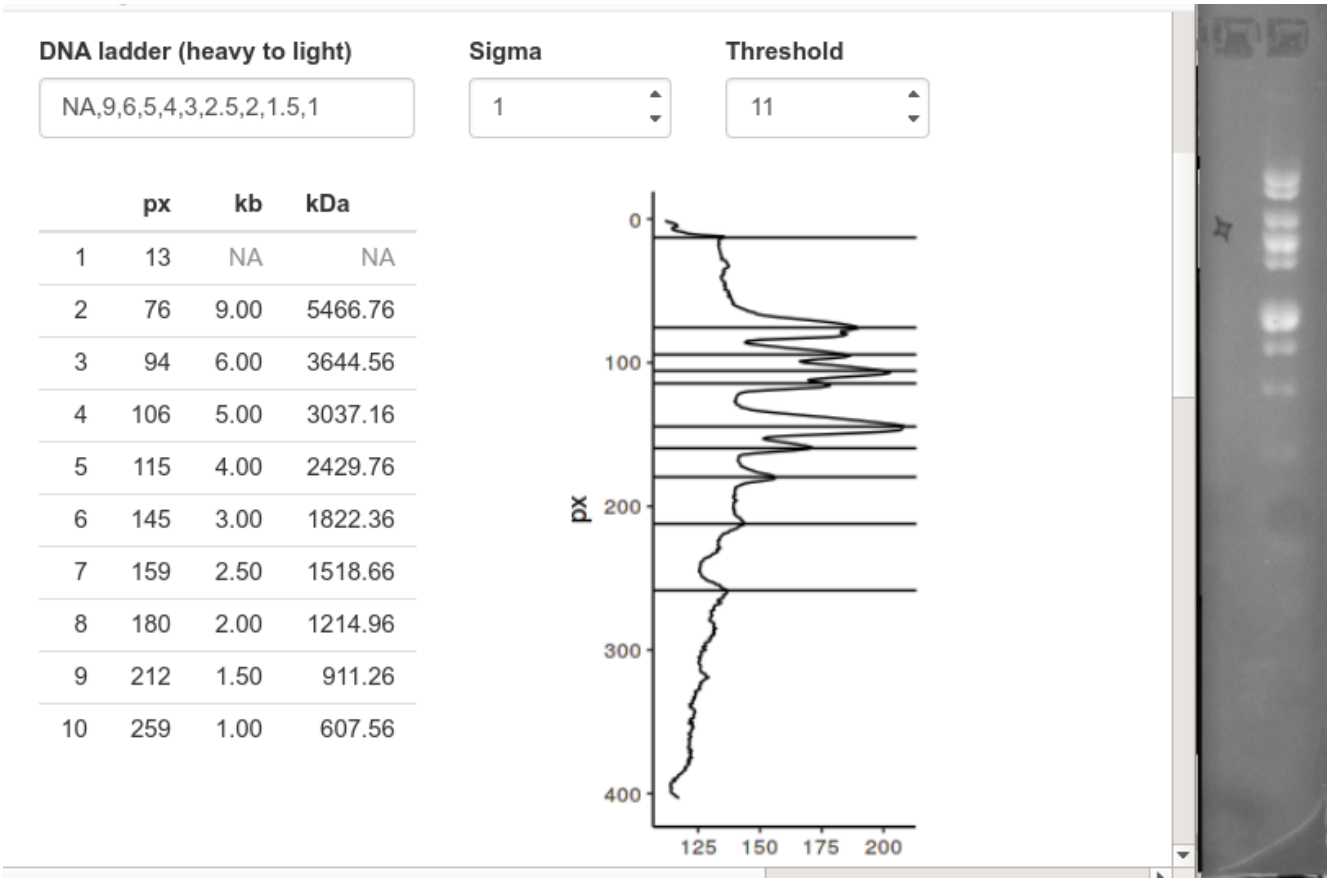
   The program provides realistic default values, but we strongly encourage you to use your own.

5. Now we are ready to get weights of DNA ladder in kb. Go to the next tab (2. Ladder peaks). First adjust the and `Sigma`, `Threshold` values (please note that you can use arrow keys to select the values) to make the lines at the plot match the peaks you see. If phantom peaks still appear, it is not a big problem (see below), but non-recognized peaks can interfere with the regression model.

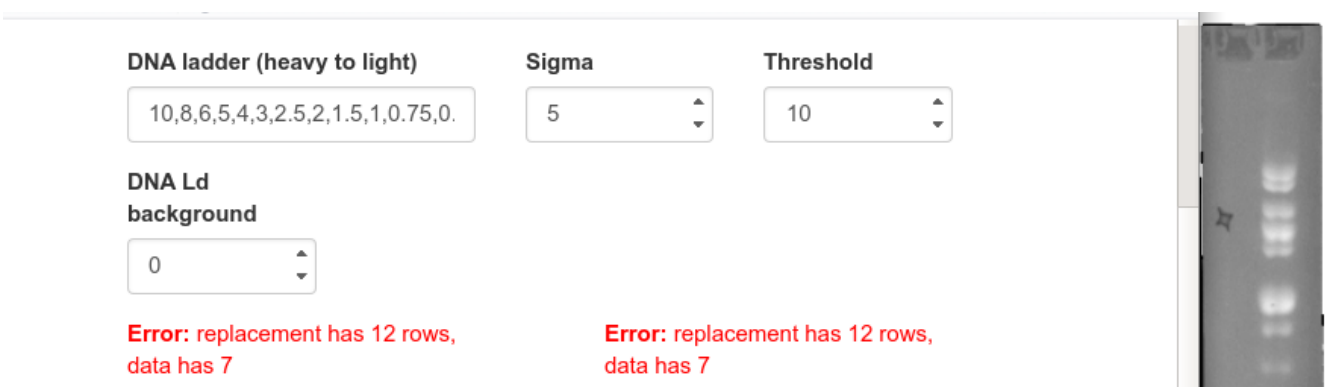
6. We recommend that at this step you keep the ladder image on screen, as we did it in the image below. By default the function tries to fit standard DNA ladder produced by SibEnzyme. Check whether it is true and make necessary adjustments.



Please note that if at the previous step there were phantom peaks, place NA at the following position into the `DNA ladder` field. These values will be omitted for further calculations. An example of such situation is shown below (an additional band is recognized near to the well).



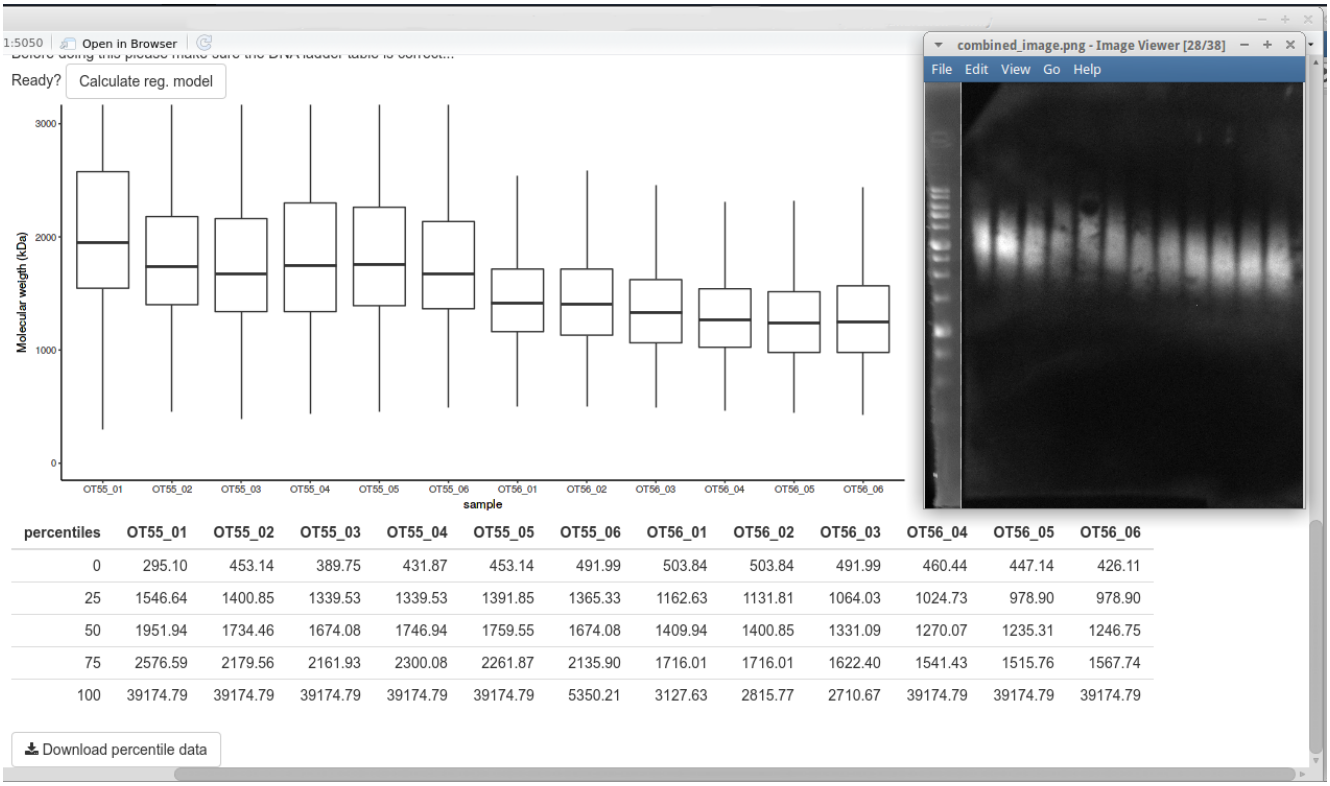
In some cases, you might see an error at this step:



If this happens, remove values from the `DNA ladder` field until the plot appears, and you are able to re-enter your values.

7. When you are satisfied with the correspondence between the real bands and those recognized by the algorithms, move to the next tab and press the `Calculate reg. model` button. Now you should see boxplots for each of the samples and two tables summarizing the corresponding numeric data (percentiles and peaks).

   In our example, the first 6 samples and the second 6 samples represented two different $[PSI^+]$ strains, and the boxplots reproduce the difference seen by naked eye correctly.



percentiles	OT55_01	OT55_02	OT55_03	OT55_04	OT55_05	OT55_06	OT56_01	OT56_02	OT56_03	OT56_04	OT56_05	OT56_06
0	295.10	453.14	389.75	431.87	453.14	491.99	503.84	503.84	491.99	460.44	447.14	426.11
25	1546.64	1400.85	1339.53	1339.53	1391.85	1365.33	1162.63	1131.81	1064.03	1024.73	978.90	978.90
50	1951.94	1734.46	1674.08	1746.94	1759.55	1674.08	1409.94	1400.85	1331.09	1270.07	1235.31	1246.75
75	2576.59	2179.56	2161.93	2300.08	2261.87	2135.90	1716.01	1716.01	1622.40	1541.43	1515.76	1567.74
100	39174.79	39174.79	39174.79	39174.79	39174.79	5350.21	3127.63	2815.77	2710.67	39174.79	39174.79	39174.79

Finally, with the button below you can download more detailed data (100 percentiles and peaks) and use it for subsequent statistical analysis.

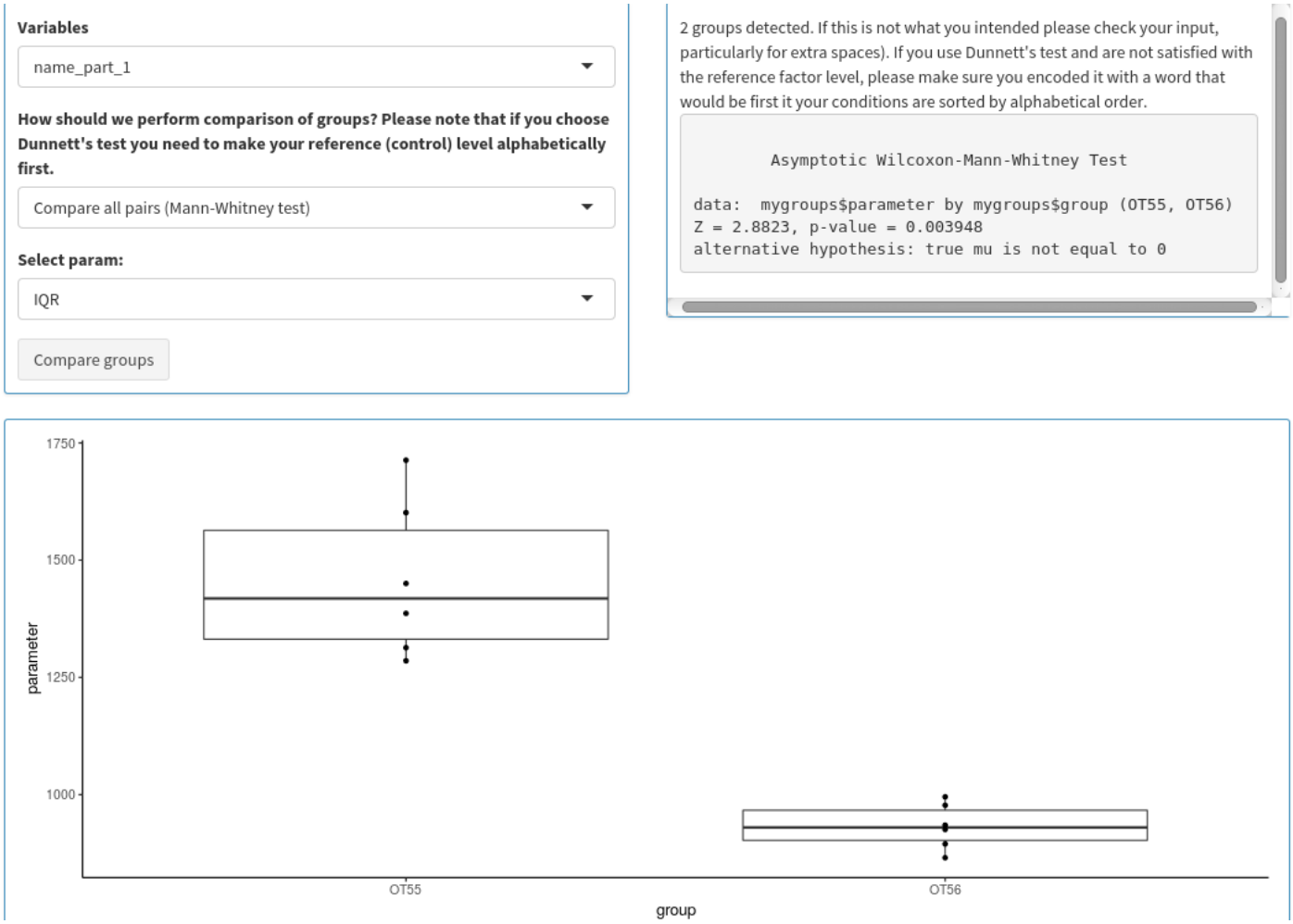
Further processing of the data

The obtained percentile and peak data data can be used for any kind of statistical analyses the user feels appropriate.

Here we provide the interface for the most obvious tests: comparison of two groups with the Wilcoxon-Mann-Whitney test and comparison of each group to the reference level with Dunn's test. For deeper analysis please download the data as a table and analyze it with your software of choice.

To perform the above-mentioned test, please navigate to the last tab (4. Sample comparison) and check if the program correctly recognized the groups of samples. If not, choose the correct part of the name.

Then use the test and the parameter to compare (the median characterizing the center of the aggregate distribution, peak optical density value or interquartile range (IQR) characterizing the spread of the distribution) and press the `Compare groups` button. Please note that if you choose Dunnett's test you need to make your reference (control) level alphabetically first.



You can now copy/save the resulting image and test results.