BioNumerics Tutorial:

Importing and processing gel images

1 Aim

Comprehensive tools for the processing of electrophoresis fingerprints, both from slab gels and capillary sequencers are incorporated into BioNumerics. In this tutorial we will focus on the import and processing of gel images. Processing tools include lane finding, normalization of the patterns, band finding, etc.

2 Sample data

As an exercise, we will import an 8-bit TIFF gel image which was generated by PFGE with the restriction enzyme **Xba-I**.

The gel image can be downloaded from the Applied Maths website: Go to http://www.applied-maths.com/download/sample-data and click on "BioNumerics Tutorial Data".

3 Creating a fingerprint type experiment

First we need to create a new fingerprint type experiment before we can import a gel image file.

1. Create a new database (see tutorial "Creating a new database") or open an existing database.

2. In the *Main* window, click on ➕ in the toolbar of the *Experiment types* panel and select ***Fingerprint type*** from the list (see Figure 1).
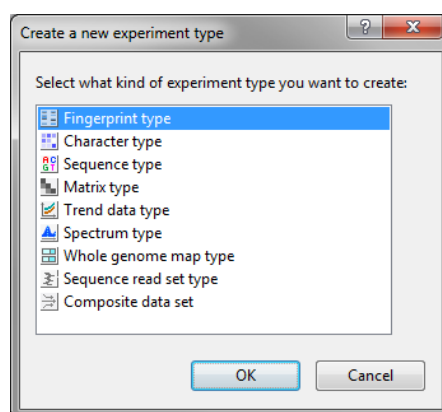

Figure 1: The *Create a new experiment type* dialog box.

3. Press *<OK>*, enter a name, for example **PFGE-XbaI** and press *<Next>*.

4. In the next window, make sure that ***Two-dimensional TIFF files*** and ***8-bit (256 gray values)*** are selected and press *<Next>*.

5. In the next dialog box, select ***Yes*** for fingerprints with inverted densitometric values.

6. Press <*Next*> to proceed.

7. In the final step, leave *No* selected for applying a background subtraction.

8. Press <*Finish*> to complete the creation of the new fingerprint type.

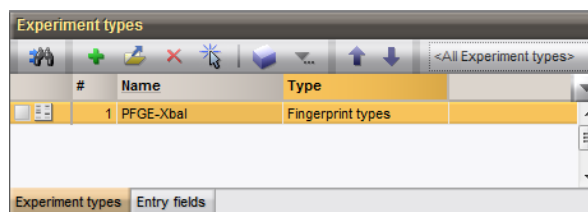The *Experiment types* panel now lists the fingerprint type **PFGE-XbaI** (see Figure 2).

Figure 2: The *Experiment types* panel.

You can still change the resolution, inversion, and background subtraction settings later when processing a gel.

4   Importing a fingerprint gel file

1. To add a new fingerprint file to the database, select *File* > *Import...* (, **Ctrl+I**) and select *Import gel file* under *Fingerprint type data* (see Figure 3).

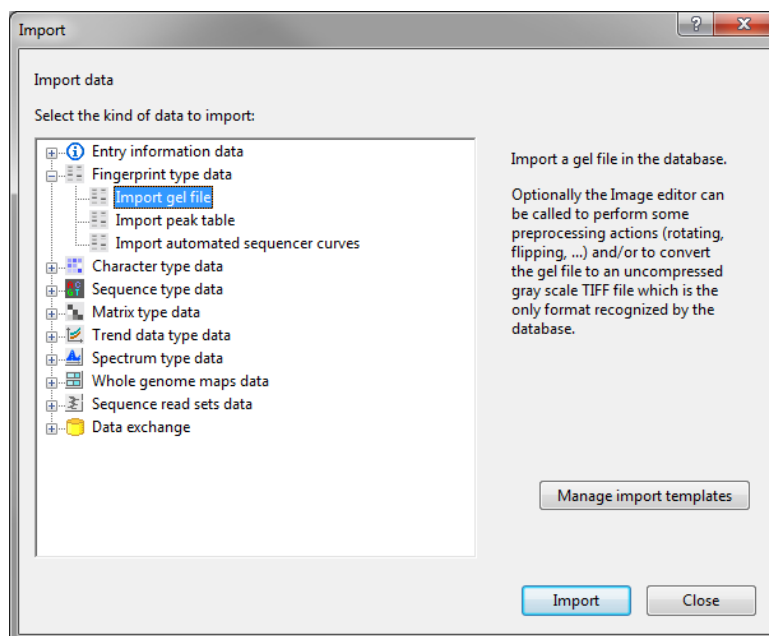**Figure 3:** Import tree.

2. Press <*Import*> to call the *Select fingerprint file* dialog box.

3. Select the file `ec-XbaI-001.tif` in the `BioNumerics Tutorial data \PFGE TIFFS` folder.

The dialog also asks if you want to edit the image by opening the image editor. Uncheck the *Open in image editor* option if you are sure the file is an uncompressed gray scale TIFF image. For the conversion to an
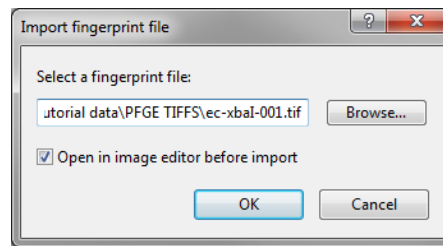
Figure 4: Select gel image.

uncompressed gray scale TIFF file make sure the option is checked.

    4. Since the example file is an uncompressed gray scale TIFF file, uncheck the option and press *<OK>*.

The gel image is now available in the *Fingerprint files* panel (see Figure 5). The file is marked with a red "N" (**N**) indicating that the image has not been processed yet.
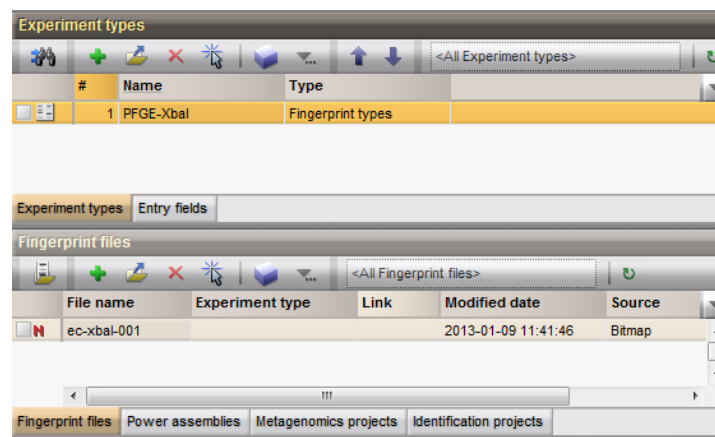

Figure 5: The *Experiment types* panel and *Fingerprint files* panel.

5 Processing a fingerprint gel file

5.1 Introduction

Before linking our fingerprint lanes to entries in our database, we must define and process the lanes in the *Fingerprint processing* window.

    1. In the *Fingerprint files* panel, double-click on the filename **ec-XbaI-001** to open the *Fingerprint* window.

    2. In the *Fingerprint* window, select *File* > *Edit fingerprint data...* (▦) to open the *Fingerprint processing* window.

    3. In the next dialog box, select **PFGE-XbaI** and press *<OK>*.

The *Fingerprint processing* window opens. In this window we are going to process our TIFF file in 4 steps:

1. **Strips** (defining lanes)

2. **Curves** (extracting densitometric curves)

3. **Normalization**

4. **Bands** (defining bands and quantification)

The steps are shown in the tabs in the bottom left corner of the window. The ◀ and ▶ buttons in the toolbar are to move back and forth, respectively. The tabs can also be used for the navigation between the different steps.

5.2 Step 1: Define strips

The imported gel image is surrounded by a green rectangle. Since BioNumerics recognizes the darkness as the intensity of a band, make sure the bands appear as dark bands on a white background (see Figure 6).

In case the bands appear as white bands on a black background, invert the values: press ⊞ to open the *Fingerprint processing settings* dialog box, check/uncheck the ***Inverted values*** check box, and press <***OK***> to apply the changes.

The first step in processing a gel is to crop the image to remove empty space, and to define the lanes.

4. Delineate the area of the gel lanes by clicking and dragging the nodes of the rectangle to adjust it. Exclude the wells from the rectangle (see Figure 6).
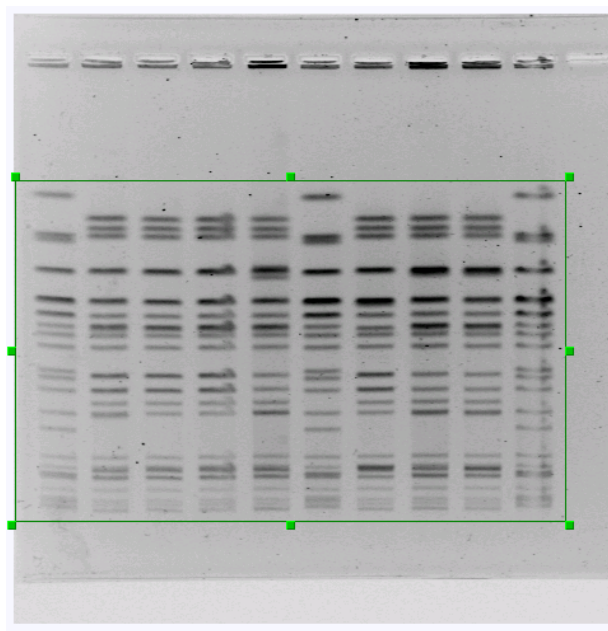

Figure 6: Area of gel lanes.

5. In the toolbar, press ⊞ to let the software search for the individual lanes.

6. Enter "10" as the approximate number of lanes and press <***OK***>.

7. In the toolbar, press ⊞ to open the *Fingerprint processing settings* dialog box.

When opened in the *Strips panel* the *Raw data tab* is selected by default.

8. Adjust the ***Thickness*** of the image strips so the splines border the edges of the bands (e.g. **33** points).

9. Press <***OK***>.

10. Adjust the horizontal position of each spline as necessary by clicking and dragging a blue node. Hold down the **Shift**-key while dragging a node to adjust the spline locally.

11. Decrease the thickness of the spline in lane **10** by clicking one of its nodes and repeatedly pressing ▚▞ or **F8**. Then move the spline over to the good portion of the lane to the left (see Figure 7).
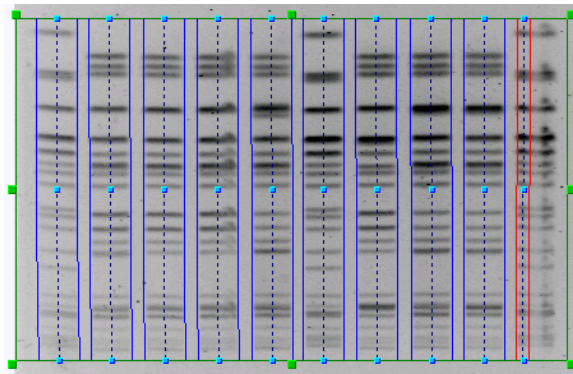

Figure 7: Adjusting the thickness and positions of the splines.

Next, we will edit the tone curve to improve the band visibility.

12. From the pull down menu, select **Edit > Edit tone curve...**.

13. Press <**Linear**>.

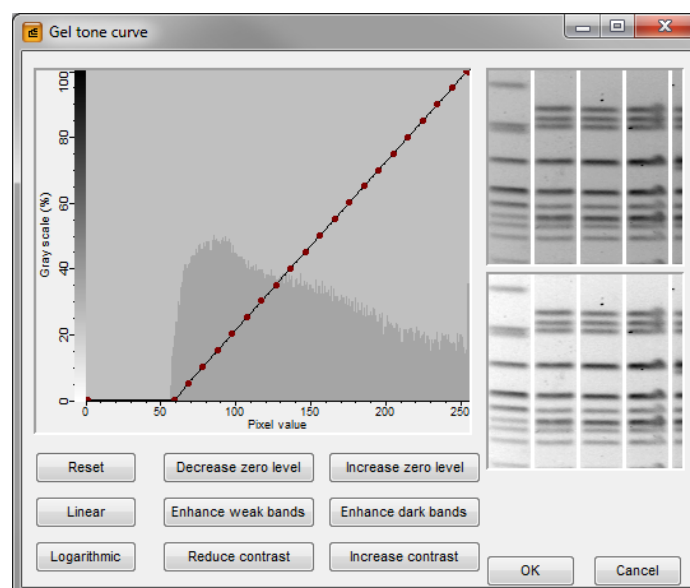The visible gray scale interval now ranges between the minimum and maximum values in the image (see Figure 8).

Figure 8: The *Gel tone curve window*.

14. Press the other editing buttons such as <**Enhance weak bands**> a few times until you have a clear and sharp image.

15. Press <**OK**> to save changes to the tone curve settings.

The gel tone curve does not change the image's densitometric values, only the way they appear in the *Fingerprint processing* window.

16. Press 🖫 to save the work already done.

17. Press ▶ to proceed to the **Curves** step or press the *Curves tab*.

5.3 Step 2: Define curves

Now that the lanes have been defined, the software can generate densitometric curves describing the optical density across the spline along each lane. The left panel shows the strips extracted from the image file, the right panel shows the densitometric curve of the selected pattern (see Figure 9). The area between the blue lines of each lane will be used to calculate the densitometric curve.

18. Select lane **3**.

Near the top of the lane in the spline there is a pinpoint spot. As a result, a thin peak appears on the densitometric curve shown on the right side of the window.

19. It might help to zoom in on the image using the zoom slider.

20. Open the *Fingerprint processing settings* dialog box again (click on the ⊞ button).

21. Change the ***Averaging thickness*** for curve extraction to **15** in the *Densitometric curves tab*.

22. Check ***Median filter*** and press *<OK>*.

In lane 3, the thin peak - resulting from the pinpoint spot - has disappeared in the densitometric curve shown on the right side of the window (see Figure 9).
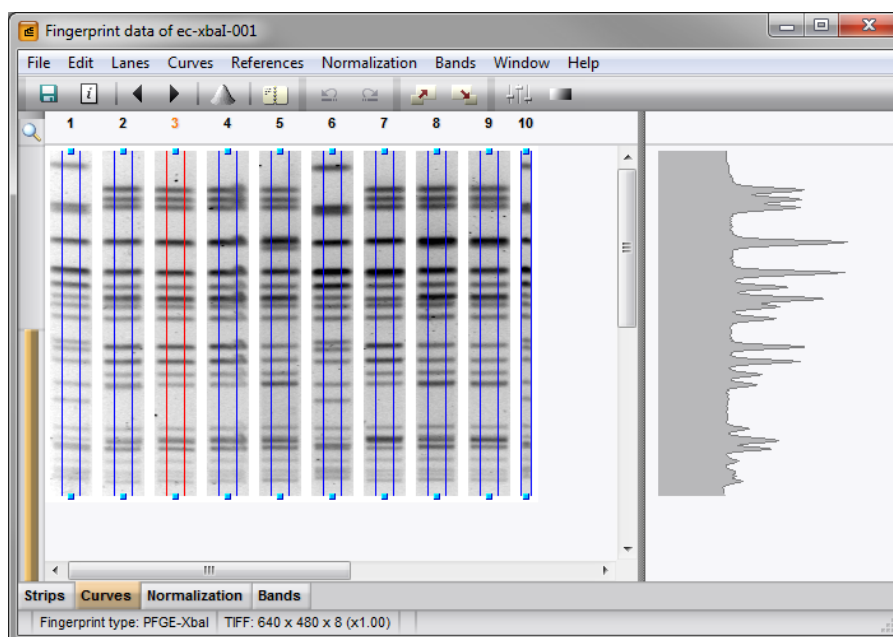

Figure 9: Increased splines and median filtering.

BioNumerics can analyze the gel image to determine the optimal settings for removing background noise from the densitometric curves.

23. Select ***Curves > Spectral analysis...***.

The following settings are shown in the *Spectral analysis* window:

1. **Wiener cutoff scale**: Determines the optimal setting for least square filtering.

2. **Background scale**: Estimation of the disk size for background subtraction.

24. Close the *Spectral analysis* window.

25. Open the *Fingerprint processing settings* dialog box again (click on the ⊞ button).

26. Check ***Apply least square filtering*** and specify a ***Least square filtering Cut off*** as indicated by the Wiener cutoff scale in the *Spectral analysis* window (use the percentage value, e.g. **1**). Least square filtering removes very small peaks from the curves.

27. Check ***Apply*** in the *Background subtraction panel* and specify a ***Background subtraction disk size*** as indicated by the background scale in the *Spectral analysis* window (use the percentage value, e.g. **14**). Background subtraction removes large background trends from the curves.
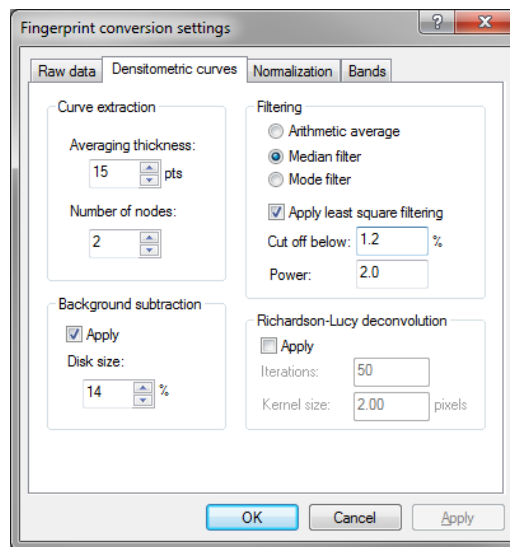

Figure 10: The *Fingerprint processing settings* dialog box: the *Densitometric curves tab*.

28. Press <***OK***>.

The background noise has been removed from the curves (see Figure 11).

Figure 11: Curve after filtering.

29. Press ![save icon] to save the work already done.

30. Press ![play icon] to proceed to the **Normalization** step or press the *Normalization tab*.

5.4 Step 3: Normalize the gel

Every fingerprint type experiment needs at least one reference system to normalize its gels. Since this is the first Xba-I gel we have imported, we need to create a reference system based on the standard pattern in this gel. We will use the standard's molecular weights to name the reference positions. Subsequent Xba-I gels, provided they contain the same standard, will be normalized with the same reference system.

31. Press ![icon] to enter the normalized view.

For now, the "normalized view" looks the same as the original view. In a first step we will define the reference lanes and the reference bands.

32. Select lane **1** and press ![icon] to assign it as a reference lane or select *References > Use as reference lane*.

33. Repeat this for lane **6** and lane **10**.

34. Choose the most suitable standard lane for creating the reference system. For this exercise select lane **6** by clicking anywhere in the lane so that the lane number turns orange.

35. Right-click on the top band in lane **6** and select *Add external reference position*.

36. Enter **582.6** and press *<OK>*.

37. Repeat the process for each band in lane **6** as shown in Figure 12.

The reference system for experiment type **PFGE-XbaI** is now defined (see Figure 13).

38. Select *Normalization > Auto assign...* (![icon]).

39. Make sure *Using bands* is selected and press *<OK>*.

40. Carefully inspect the assignments made (see Figure 13).

41. If a band assignment is incorrect, select the band and press the **Del**-key.

42. To assign a reference band manually, first click on the reference position tag, then hold the **Ctrl**-key and click on the reference band to assign it to that reference position.

43. To update the normalization based on the band assignments, select *Normalization > Update normalization* (![icon], **Ctrl+U**).

44. Press ![play icon] to proceed to the last step. Alternatively press the *Bands tab*.

5.5 Step 4: Define bands

If you want to use the curves to compare the patterns, no bands need to be assigned and this last step can be skipped. If you want to compare the patterns using bands, you will need to assign bands in the sample lanes in this last step. Usually, assigning bands in the sample lanes is done first with the software's automatic band search, followed by manual corrections. Some trial and error might be required to find the best settings for the automatic band search.

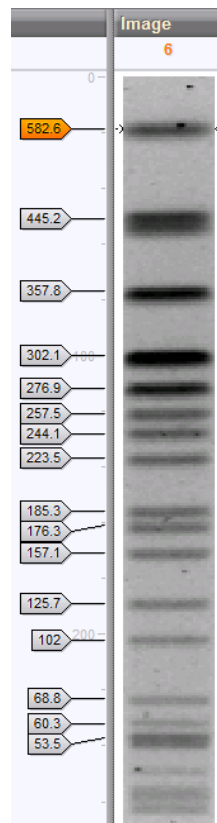45. To automatically search for bands, press ![icon] or select *Bands > Auto search bands*.
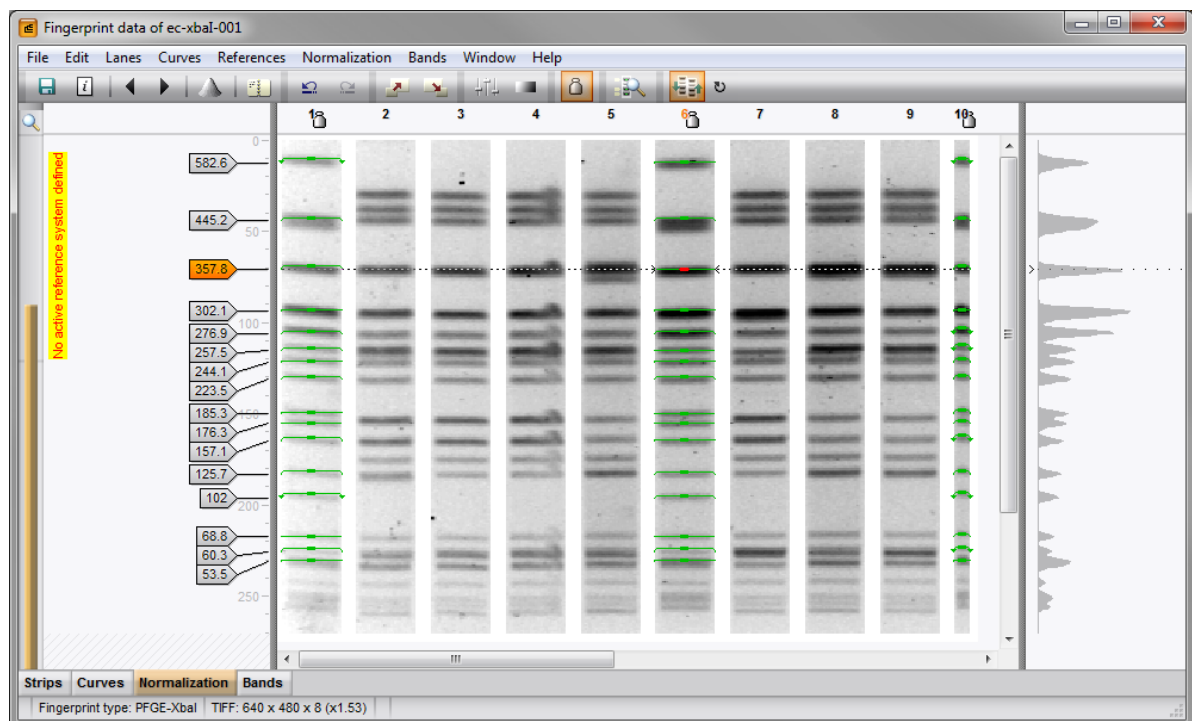
Figure 12: Reference system.

Figure 13: Reference positions assigned.

In the *Band search* dialog box, the currently selected lane is shown along the bottom (see Figure 14).

46. To scroll through other lanes, press the < and > buttons on the left and right sides of the curve.
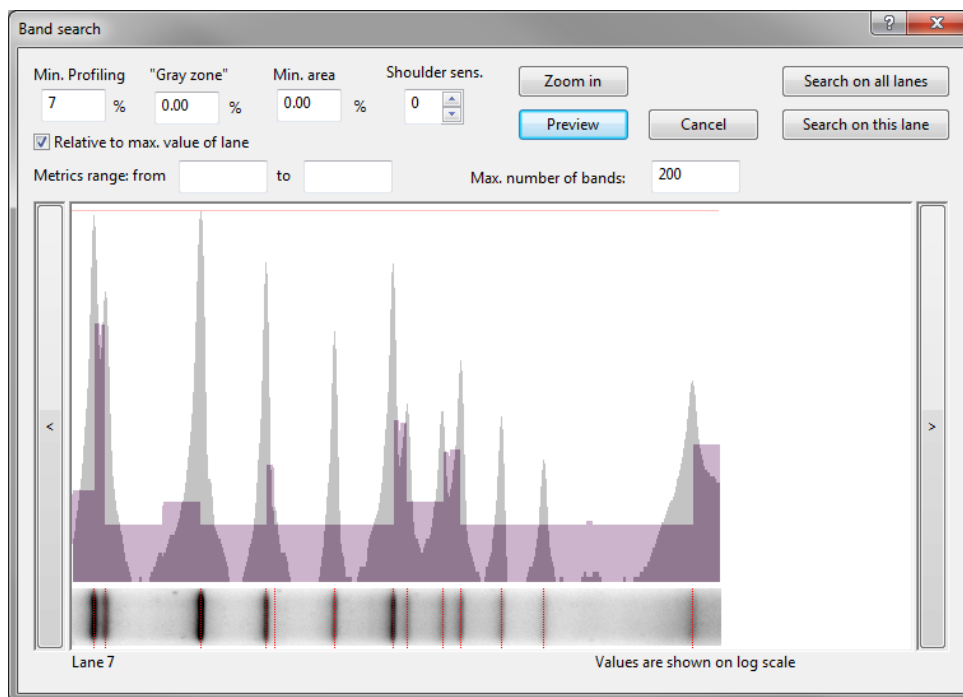
Figure 14: The *Band search* dialog box.

47. Enter **5** for *Min. Profiling*, **2** for *Gray zone*, and press *<Preview>*.

The red lines across the lane at the bottom of the window indicate which peaks will be assigned bands with the current settings. The purple area along the curve indicates the minimum profile above which a band is assigned, while the blue area indicates the gray zone in which bands are considered "uncertain".

48. Press *<Search on all lanes>* to execute the band search with these settings.

Bands that are found are marked with a green horizontal line, whereas uncertain bands are marked with a small green ellipse (see Figure 15). If you see any incorrect band assignments you can edit them manually:

- To add a band, hold down the **Ctrl**-key and click on the spot. The cursor automatically jumps to the closest peak; to prevent this, hold down the **Tab**-key while clicking.

- To select a group of bands, hold down the **Shift**-key and click while dragging the mouse pointer diagonally across the bands.

- To delete one or more selected bands, press the **Del**-key.

- To mark a band as uncertain, click on the band and select *Bands > Mark band(s) as uncertain* or press **F5**. To mark a band as certain, click on the band and select *Bands > Mark band(s) as certain* or press **F6**.

49. After you are satisfied with the band assignments, press 🖫 to save the file.

50. The program may prompt with the following question: "The resolution of this gel differs considerably from the normalized track resolution. Do you wish to update the normalized track resolution?" If the question appears, answer *<Yes>*.

51. Exit the *Fingerprint processing* window by selecting *File > Exit*.

52. The software asks: "Settings have been changed. Do you want to use the current settings as new defaults?" Select *<Yes>* so that the settings used for this gel will be saved in the fingerprint type settings.
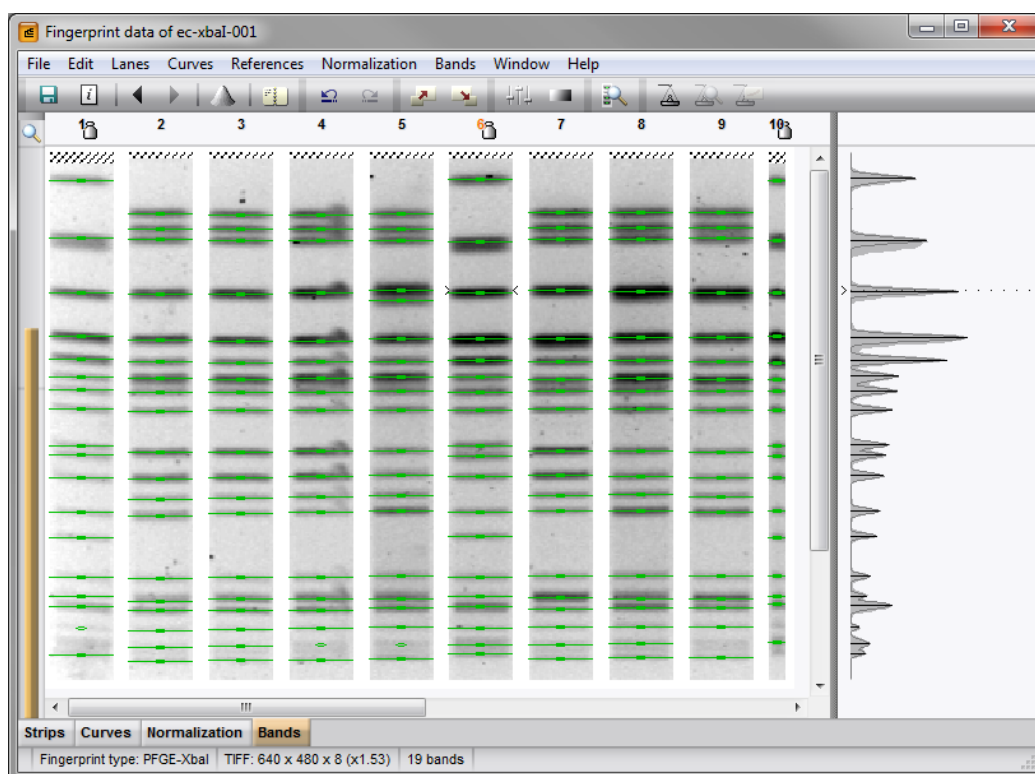
Figure 15: The *Bands tab*.

Congratulations! You have processed your first gel. The reference system and fingerprint settings that are saved with the fingerprint type experiment will make future XbaI gels much faster to process.

6   Linking fingerprint data to entries

Although we have created individual fingerprint lanes from our gel image, the software does not know which lanes correspond to which entries in the database. Our next task is to link the fingerprint lanes to the entries in our database.

1. In the *Fingerprint files* panel, double-click on the filename **ec-XbaI-001** to open the *Fingerprint* window (see Figure 16).

2. Select lane **2** and select *Database > Link lane...* or press ⬆.

3. In the dialog box enter **EC001** and press *<OK>*. Confirm the creation of the new entry.

You can also link a lane to an existing database entry by dragging the gray arrow icon to the entry key in the database window. When a lane is linked, the icon becomes purple: ⬆.

4. Continue linking the remaining non-reference lanes from **ec-XbaI-001** to new database entries as shown in Figure 16.

> You can also let the program create new entries and link the gel lanes automatically by selecting *Database > Add all lanes to database*. All unlinked lanes will be added as new entries to the database, with the gel lanes linked to these entries. The entries will be assigned a unique key.

5. After linkage, close the *Fingerprint* window and open the gel strip for one of the entries in the database by clicking on a colored dot in the *Experiment presence* panel.
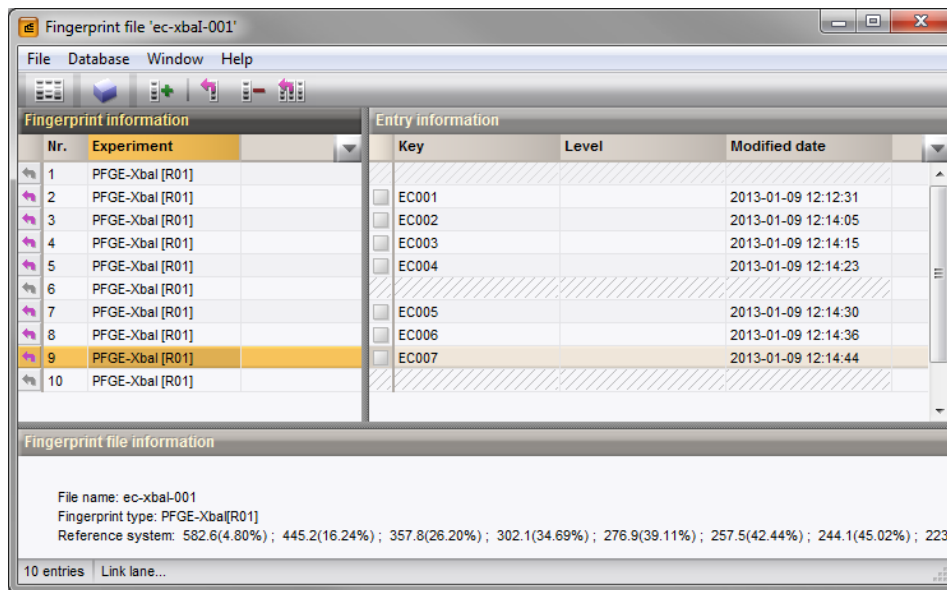
Figure 16: Fingerprint file with linked lanes.

The card is displayed in raw mode (i.e. not normalized). The band sizes are shown as relative distances from the top (see Figure 17).
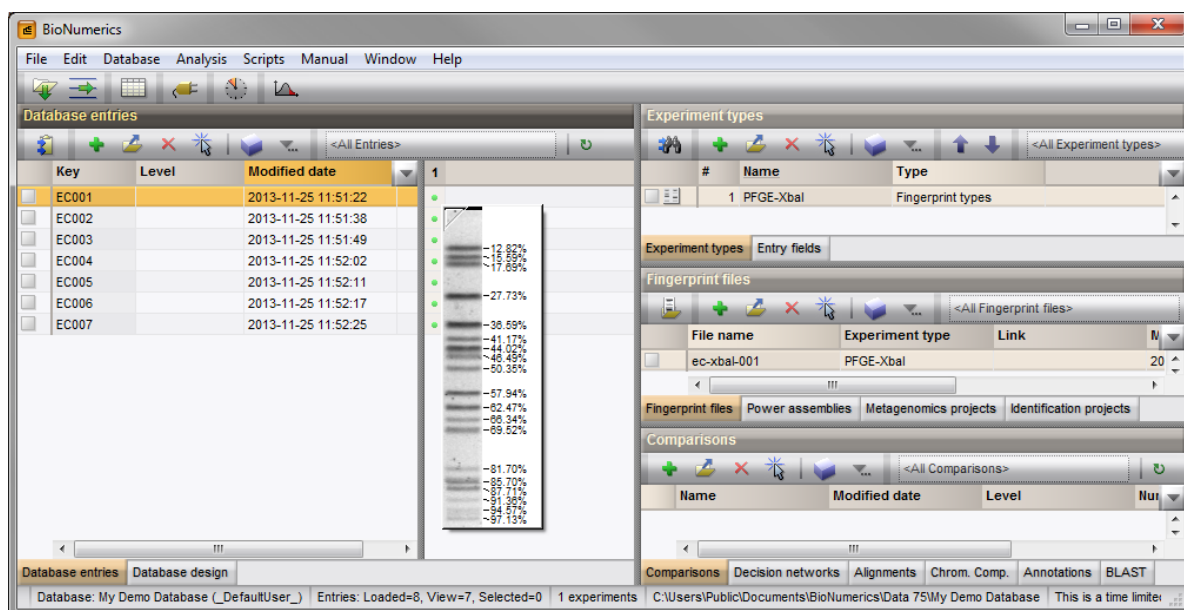

Figure 17: Fingerprint type experiment card: raw mode.

6. Close the card by clicking in the small triangle-shaped button in the upper left corner.

7 Fingerprint type experiment settings

1. Open the fingerprint experiment type **PFGE-XbaI** by double-clicking on the experiment type in the *Experiment types* panel.

The *Fingerprint type* window shows the defined reference positions in relation to the distance on the pattern in percentage. The reference system is called **R01**. The panel left from the reference system is still blank:

the fingerprint still misses a standard pattern. We will link a **Standard** pattern (e.g. lane **6** of **ec-XbaI-001**, i.e. the one used for defining the reference system) to the **PFGE-XbaI** fingerprint type as follows:

2. Close the *Fingerprint type* window.

3. In the *Fingerprint files* panel, double-click on **ec-XbaI-001** to open the *Fingerprint* window.

4. In the *Fingerprint* window, add lane **6** to the database by selecting it and pressing ![icon].

5. In the dialog box, enter "REF" and press *<OK>*.

6. Select *<Yes>* to create the new entry in the database.

A new entry **REF** is created with the pattern from lane **6** of **ec-XbaI-001** linked to it.

7. Close the *Fingerprint* window.

8. Open the fingerprint experiment type **PFGE-XbaI** by double-clicking on the experiment type in the *Experiment types* panel.

9. Press ![icon] next to **Standard** in the *Settings panel* and drag it over to the **REF** database entry.

The standard pattern is now displayed in the panel next to the reference positions, and the database entry key **REF** is indicated as the **Standard** (see Figure 18).
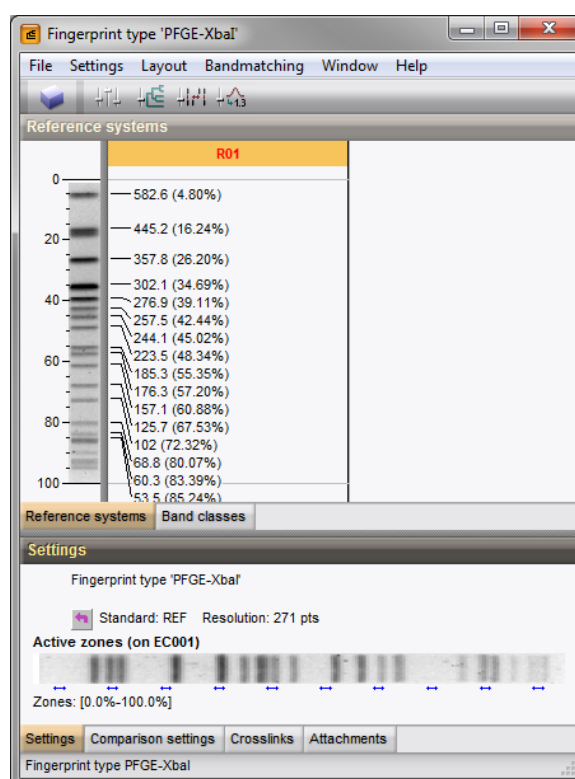

Figure 18: The *Fingerprint type* window.

If a standard pattern is assigned to a fingerprint type, this standard pattern is shown in the *Normalization tab* of the *Fingerprint processing* window (see Figure 13) to make visual assignment of bands to the reference positions easier. The choice of a standard has no influence on the normalization process since it is only used as visual aid.

One more step is necessary before we can analyze our fingerprint patterns. Since there are gaps present between the reference system positions, we must tell the software how to convert other band positions into

metrics (e.g. molecular weights). We will use the reference system to construct a calibration curve which translates all band positions into metrics.

10. In the *Fingerprint type* window, select **Settings** > **Edit reference system** or double-click on **R01**.

The *Fingerprint Reference system* window appears with the message: "Could not calculate calibration curve. Not enough markers."

11. Since the molecular weights were already entered as names for the reference positions, we can copy these molecular weights by selecting **Metrics** > **Copy markers from reference system...**. Confirm the action.

12. Designate a metric unit with **Metrics** > **Assign units...**, enter **kb** and press <**OK**> (see Figure 19).
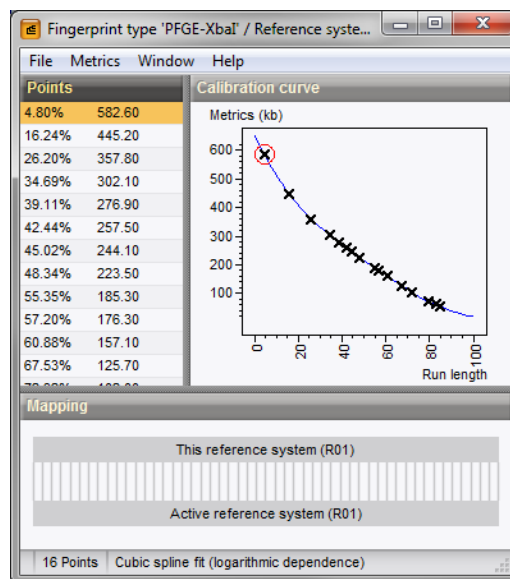

Figure 19: Calibration curve calculated.

13. Close the *Fingerprint Reference system* window, and close the *Fingerprint type* window.

The fingerprint type **PFGE-XbaI** is now defined and configured, and one gel has been added to the database.

14. Open a gel strip for one of the entries in the database by clicking on a colored dot in the *Experiment presence* panel.

The cards are now displayed in normalized mode (see Figure 20). Band sizes are shown as molecular sizes based on the regression curve calculated in the previous step.
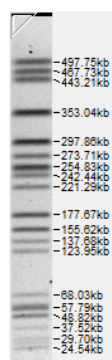

Figure 20: Fingerprint type experiment card: normalized mode.

15. Increase or decrease the size of the card using the keyboard by pressing the numerical "+" key (increase) or the numerical "-" key (decrease).

16. Close the card by clicking in the small triangle-shaped button in the upper left corner.

8 Importing and processing a second gel file

A second gel file, `ec-XbaI-002.tif`, which was generated by PFGE with the restriction enzyme **Xba-I** is present in the `BioNumerics Tutorial data \PFGE TIFFS` folder. For this second PFGE-XbaI gel, you can use the same fingerprint type, reference system, and conversion settings used for the first gel.

Because the same reference system is present in the **ec-XbaI-002** gel, **you can skip the assignment of the reference positions**: in the third step you just have to assign the reference lanes (lane 1, 4, 7 and 10), execute *Normalization* > *Auto assign...* ( ) and inspect the assignments made.

9 Conclusion

In this tutorial you have seen how easy it is to import and process gel images in BioNumerics. You can now start applying comparison functions such as band matching, clustering, etc. on the data. More information about these functions can found in the analysis tutorials on our website.