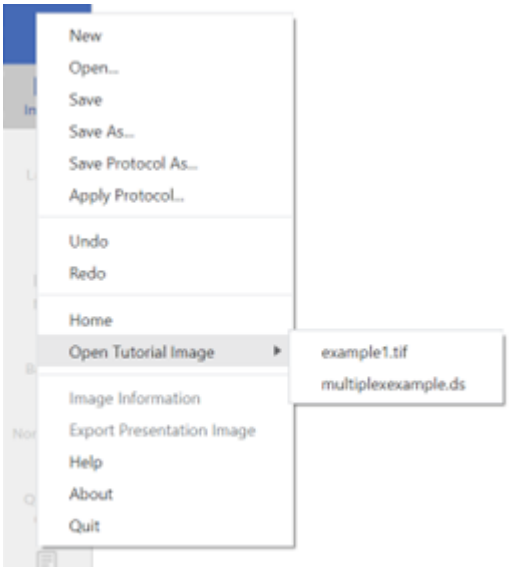
Phoretix 1D Image Analysis Software User Manual

Introducing **Phoretix 1D!**

Whilst Phoretix 1D is agnostic image analysis software and can be used to analyse images derived from many different types of experiment, this user guide will be focused on the analysis of gel and blot images.

To facilitate training of new users, the software comes pre-loaded with two example images which can be accessed from the main menu at the top left of the software window. Click the following icon to access the main menu:

And hover your cursor over **Open Tutorial Image** to be presented with two tutorial images:



example1.tif and multiplexexample.ds. Example1.tif is a singleplex 14-lane blot image and multiplexexample.ds is a 20-lane multiplex image.

Main menu bar

The Main menu bar, located at the top left of the main window, provides several commands not available through the main toolbar modes:

New

Opens a new instance of the software to allow you to analyse an additional image file, in parallel, in a new window.

Open

Allows the user to open image files for analysis. The software supports all industry-standard image formats:

.TIF/.TIFF/.GEL

> *The software supports 8-bit and 16-bit (recommended) uncompressed TIFF images. Multi-page TIFFs are supported and each page is interpreted as a "channel" (all of which must have the same dimensions and pixel format).*

.JPG/.JPEG/.PNG/.BMP

> *While not recommended because of their low bit-depth and (sometimes) compression artefacts, these image formats can be still be analysed.*

Save

Saves the current project so that it can be reopened to continue or repeat analysis of an image.

Save As

Saves a copy of the current project so that it can be reopened to continue or repeat analysis of an image without effecting the original saved copy.

Save Protocol As

Available in compatible modules only.

Saves the current analysis parameters as a protocol file so that they can be applied to other projects. More information on protocols can be found in the protocols section of this guide.

Apply Protocol

Available in compatible modules only.

Allows the user to apply a previously saved protocol file to the current project by selecting the protocol file from the file explorer.

Undo

Undoes the last action of the user on the current project. This function can also be accessed by clicking the following icon in the main window:

Redo

Redoes the last action of the user on the current project. This function can also be accessed by clicking the following icon in the main window:

Open Tutorial Image

Opens the built-in tutorial images in a new instance of the software for analysis.

Export CSV

Exports a .CSV file containing the data from the image currently displayed in the results window

PDF Report

Exports a PDF report from the current project

Image information

Displays technical information about the currently opened image. These parameters can also be exported from this window in either a text file or .csv file.

Export Presentation Image

Allows the user to export a copy of the currently open image file for use in presentations, publications etc. From this screen the user can crop the image, choose whether to highlight areas of saturation within the image and control the resolution of the exported image. Images for presentation can be saved in .png, .jpg or .bmp file formats.

Help

Displays this user manual.

About

Displays the current software version.

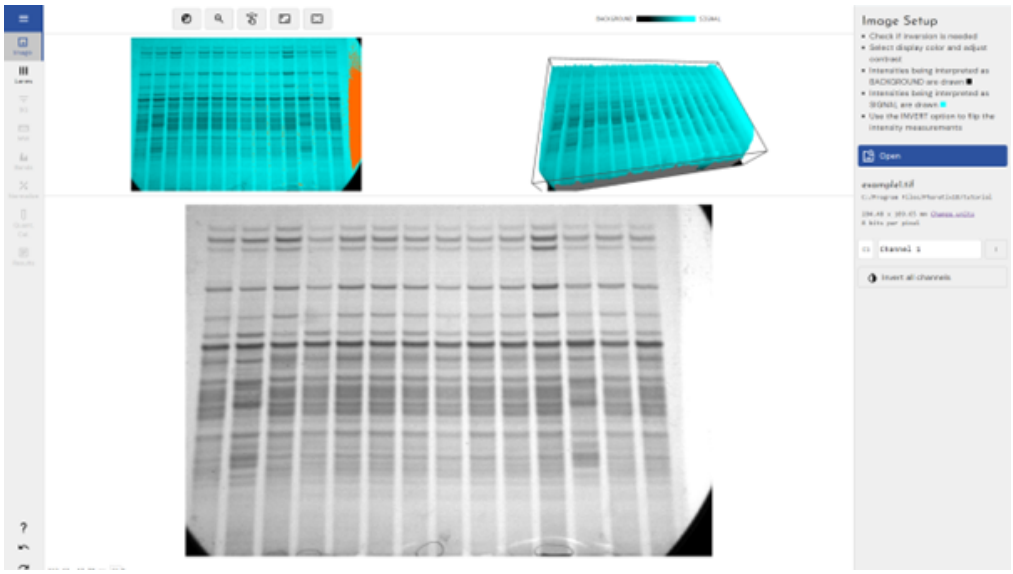
Manage License

Opens up the licensing window, where users can check their current licensing permissions and update their license key if necessary.

Quit

Closes the software.

Image Setup Window



Use the Image Setup to locate your images and inform the software how to correctly interpret and present them.

The first step in your analysis process is to load your images and make sure that the software is interpreting the data they contain correctly.

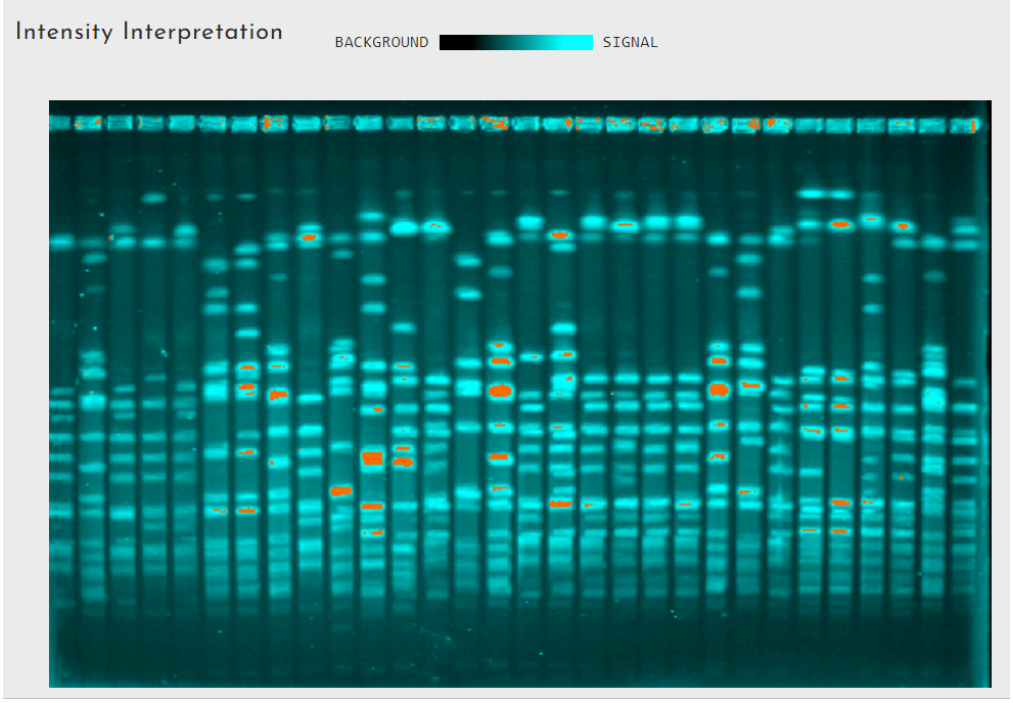
The single most common source of confusion and dysfunction in software that processes scanned image data is to mis-interpret background for signal. It can be tricky to confirm from a visual inspection alone whether the values are being interpreted correctly because more often than not, a false-color display is used to present the image.

To combat this confusion, we've developed tools in the image window to aid you in this critical first step.

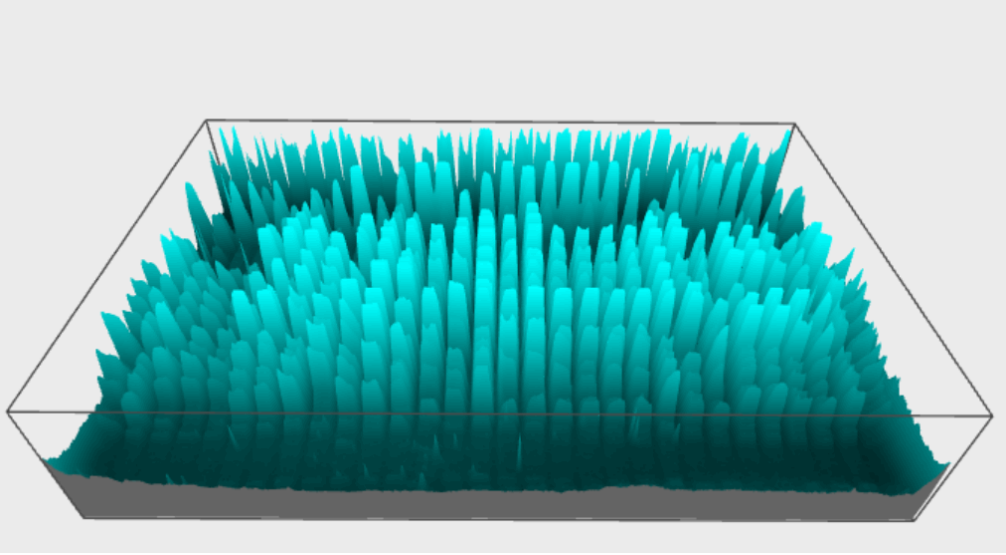
Image Displays

There are 3 image displays in the image setup window.

In the top-left corner, we see a 2D false-color representation of the data. This is referred to as the "image view" in various sections of this user manual:



In the top-right, the same image but displayed in 3D. These 2 displays visualize the magnitude of the image as it is currently being interpreted. The false color scale goes from black to bright cyan. Background data should be drawn in black, and signal in cyan. In the 3D view, bands should be the "peaks".



If these displays show the opposite - signal data in black and background in cyan, this must be corrected by inverting the image.

Image Setup Toolbar

The Image Setup window contains a toolbar with the following buttons:

 Change Display and Contrast

This tool allows the user to adjust the colour, gamma and contrast of the current image. The circular arrow button to the right of the gamma slider resets the gamma value to 1.0. The “full” button displays the full contrast range of the current image, the “auto” button allows Phoretix 1D to automatically detect the optimal contrast settings or display and the “manual” button allows the user to manually enter low and high contrast values.

 Zooming an image

This tool allows the user to zoom in and out of the main image display in the bottom section of the image setup window.

To use this tool:

- Left-click the zoom button to select.
- Position the pointer over the area you want to zoom in the main image display.
- Left-click to zoom into that area or right-click to zoom out
- Alternatively, left-click and drag across an area to zoom into that area and right-click to zoom out

 Pan

Allows the user to move the image when zoomed in. Left-click on the tool to select and left-click and drag on the zoomed in image to move around without having to reset zoom level.

 Reset Image Zoom

Resets the current image zoom to the default, showing the image in it’s original size.

 Toggle Zoom

Creates a full-screen, pop-out window of the 3D image view which allows manipulation. Left-click, hold and move the mouse to rotate the 3D view. To zoom in and out of the 3D view, use the scroll wheel on your mouse (if present). Right-click on the 3D view to either copy the image to the clipboard to paste into another application or select “save to file” to save a PNG copy of the 3D view.

Channel Properties

On the right-hand side of the Image Setup window, you'll see the following options:

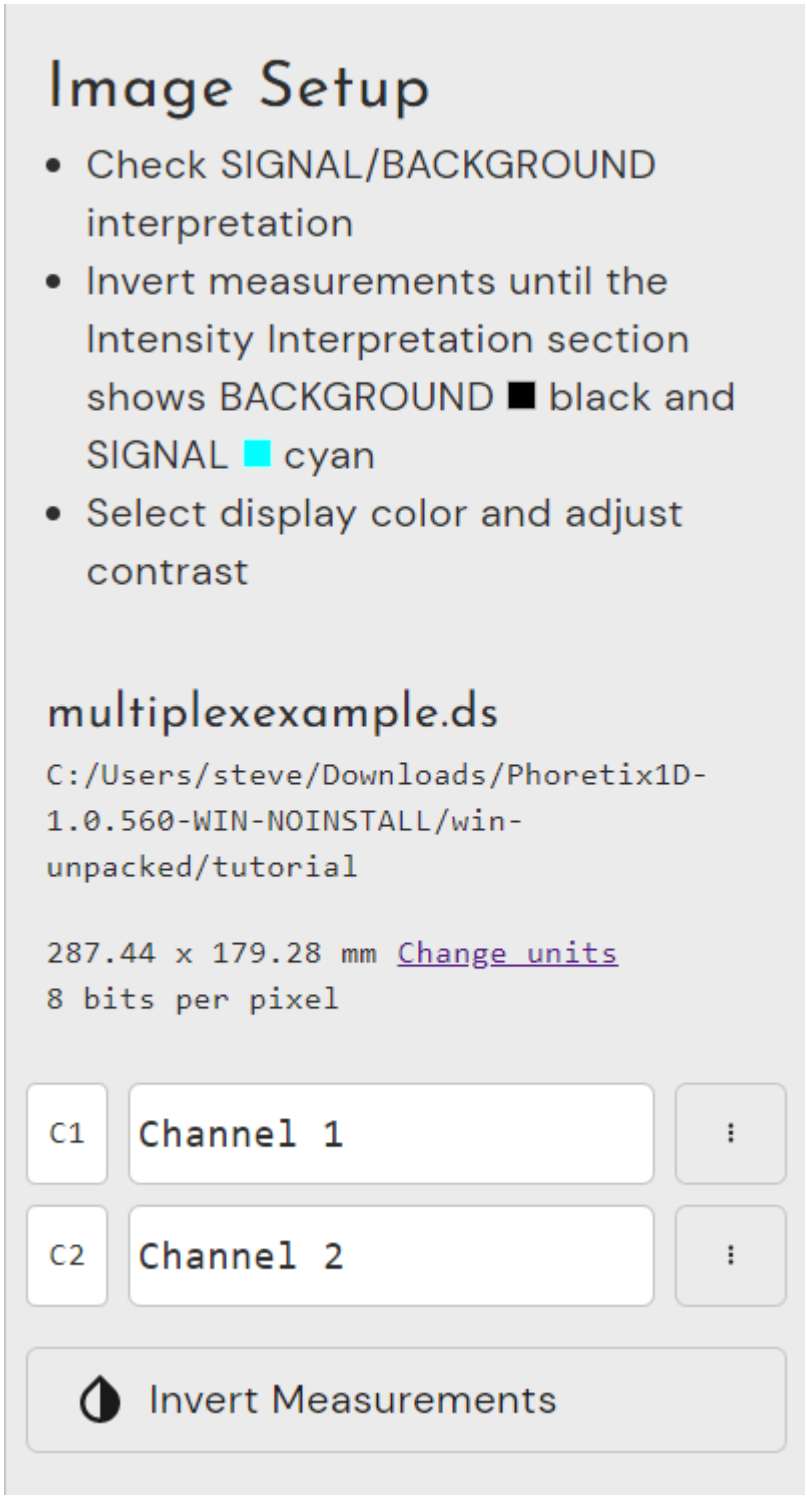
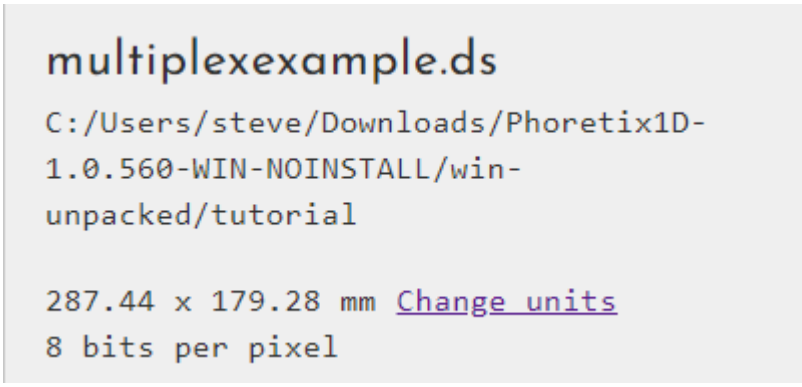


Image Name and Size

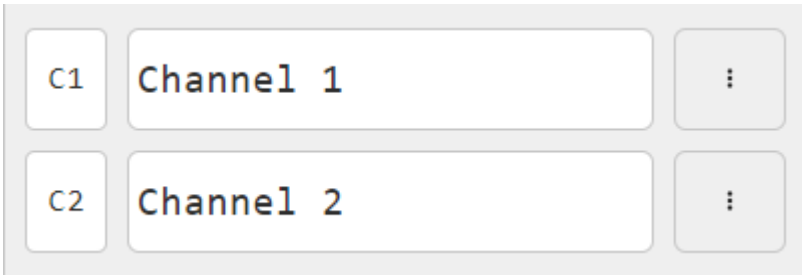
This section displays the name of the image, its file location, size and bit-depth.



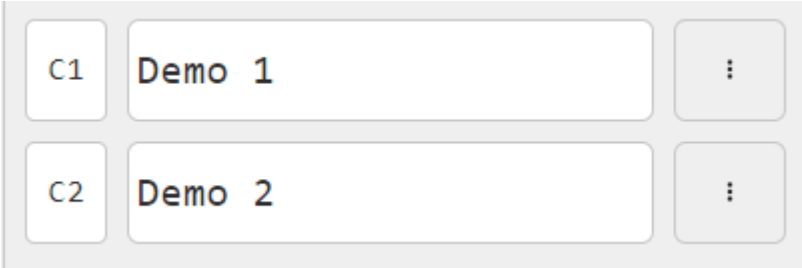
The units for image size can be changed by left-clicking on "Change units" with millimeter (mm), centimeter (cm), inches (inch) and pixels (px) supported.

Channel Name

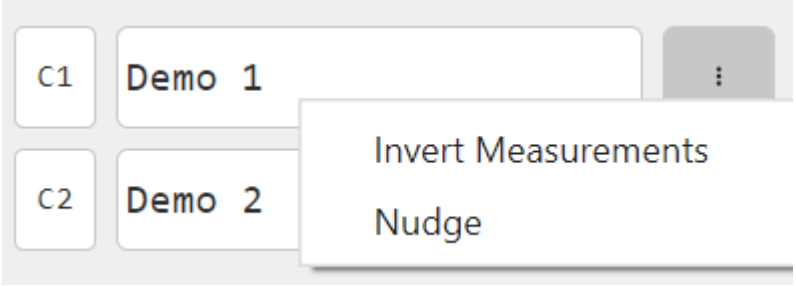
This sections displays the current channels in the open image. For a single channel image this will only show one channel, for multichannel images it will show all channels.



Channels are given numbered names by default, however the user can rename these channels by clicking within the channel name box and typing into the box like so:



To the right of the channel name box is a channel specific properties menu which can be access by left-clicking three vertical dots button:



Invert Measurements (Local)

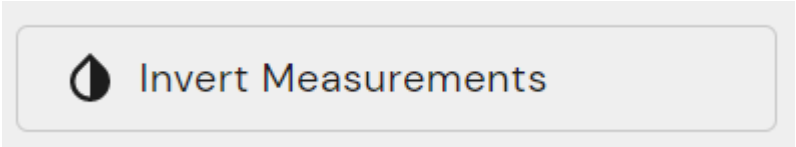
This option inverts the measurements for that particular channel, use this option if the bands in your channel are displayed as troughs instead of peaks.

Nudge

This option is only available for multichannel images and allows the images on each channel to be moved slightly (or nudged) for better alignment.

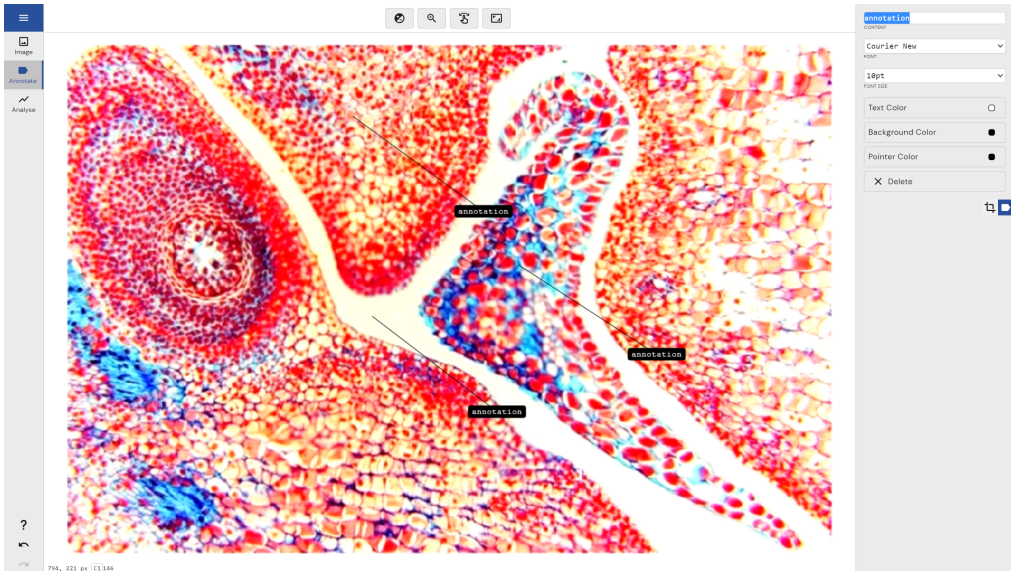
Images can be nudged horizontally (along the X plane) or vertically (Y plane) by either 1 or 10 pixels at a time by left-clicking the corresponding arrow button.

Invert Measurements (Global)



The invert measurements button below the channel names inverts the measurements for all channels in the image. Use if all channels are displaying bands (or signal) as troughs instead of peaks.

Annotations



With annotations you can mark up your images with text based tags that can draw attention to a particular area. To annotate, move into Annotations mode and click-and-drag over the image. The "mouse down" point will be the tip of the arrow, and the label will be placed based on where you move during the drag. Once placed, annotations can be easily moved. Drag the "tip" of the arrow to move just the tip. Drag the label to move the label but leave the tip pointing at the same location. Drag the "stem" of the arrow to move the whole annotation.

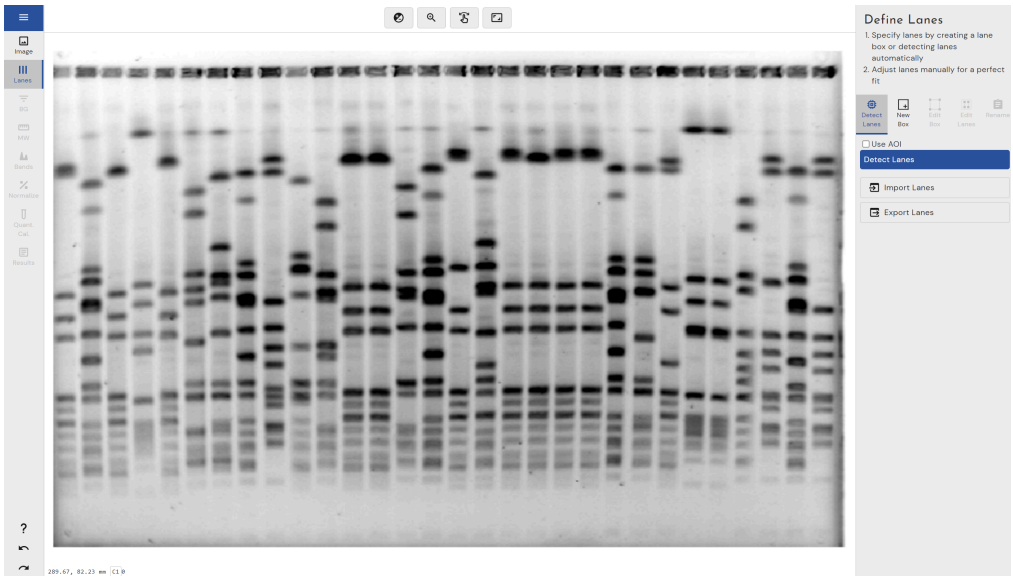
Annotations will appear on all image displays globally so long as they are "enabled" using the small toggle button at the bottom of the main sidebar.

Annotations can be optionally included in exported images - both via the "Export Presentation Image" function and PDF Reports.

Customization

Annotations have a small set of customizable properties: the actual content, font, and font size. You can also choose the color of the label foreground, background, and arrow. All customizations are applied to the "current" annotation and stored as the base for all future annotations.

Lane Mode



Use the Lane Mode to define the positions and boundaries of each lane in your gel

The tools in this mode inform Phoretix 1D where the lanes in your gel are located. Lanes must be expressed vertically and grouped horizontally into a “lane box”.

The Lanes Mode interface is split into sub-modes: Detect Lanes, New Box, Edit Box, Edit Lanes, and Rename.

Most of the time you will only have 1 set of lanes - one “lane box” but to handle multi-tiered gels and other edge cases (like placing multiple gels onto a scanner at once) you can create multiple lane boxes and fine-tune each independently.

It’s important to define the lanes correctly at the start of analysis since changing them later can affect the results.

Detect Lanes

In this mode, will attempt to automatically detect the lanes in your gel using it's built-in proprietary automatic detection algorithm.

By default, it will scan the whole image to detect lanes however, if you only wish to detect some of your lanes or exclude any areas of the gel, you can tell where to look for lanes by defining an area of interest (AOI).

If you wish to define an AOI, check the "Use AOI" box to generate a purple box on your image. Adjust the size of this purple box using

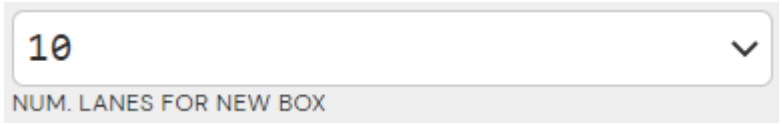
the white square handles to define your intended AOI.

Once this stage is complete (or if you don't wish to define an AOI and allow automatic lane detection on the whole image) click the detect lanes button:

> *Please note, automatic lane detection only works on a single channel. If using a multichannel image, please select one channel before attempting to use automatic lane detection.*

New Box

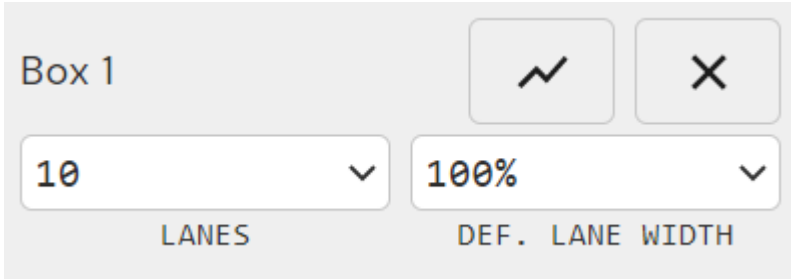
This tool allows the user to manually draw a box around their lanes to define them. Select the number of lanes your gel/blot has in the drop down menu:



and left-click, hold and drag your cursor over your image to draw the box. Lanes will be automatically equally spaced within the box.

If you have a multi-tier gel or bot, you have the option to draw multiple boxes on your image of differing sizes and lane numbers.

Once you've created and placed your box/boxes you'll notice on the right hand side of the window they begin to appear:



If you wish to further sub-divide your created box, click the subdivide button:

This will allow you greater freedom in manipulating the shape of the box by giving you more anchor points to adjust.

If you wish to delete the box, click the delete button:

After a box has been created you can change the number of lanes within it by using the lanes drop down box:

Or change the width of the lanes (but not the box) by using the Def. Lane Width drop down:

Lane width is expressed as a percentage of the total width of the lane box, for example a box with 10 lanes at 100% width will have no gaps between lanes, at 50% width will have spaces equal to the lane width between the lanes.

Below box properties you have two options for using saved lane setups:

Import lanes allows the user to import the lane configuration from a previous experiment, saving time when manually creating lanes for experiments where the lane settings remain constant.

Export lanes allows the user to export the current lane configuration as a lane object file to be imported into future experiments using the import lanes button.

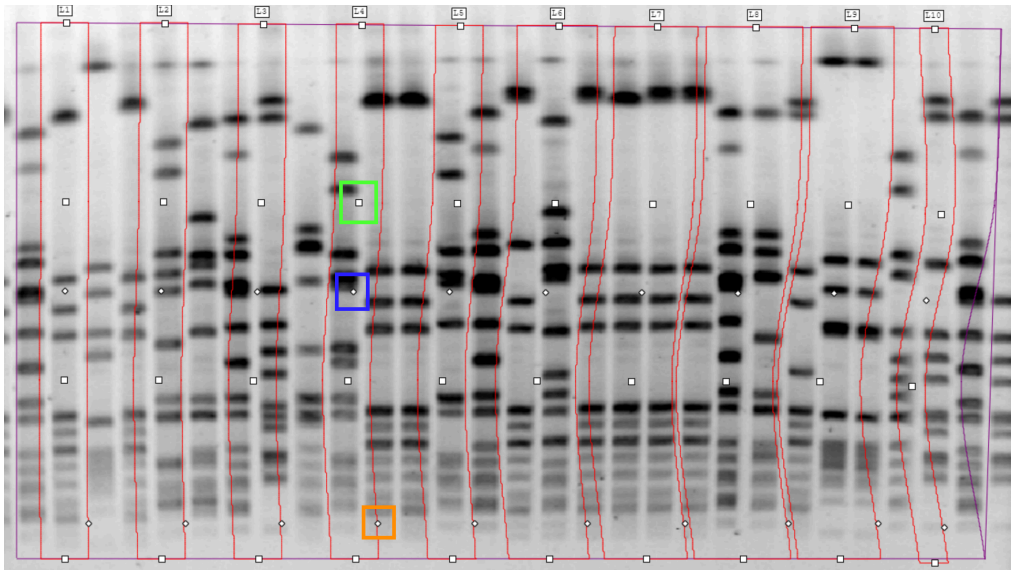
Edit Box

Entering this mode allows the user to edit the lane box. More specifically, it allows the user to accomodate for warping of lanes within the gel or lanes that haven't run perfectly vertically.

To edit the lane box, left-click, hold and drag on the square nodes on the left or right edge of the lane box.

Edit Lanes

Entering this mode allows the user to directly edit the lanes within the lane box, again allowing the user to accomodate for any warping of lanes or to manually adjust the width of individual lanes.



To bend lanes, left-click, hold and drag the squares located around the edge of the lane box (indicated by the green box).

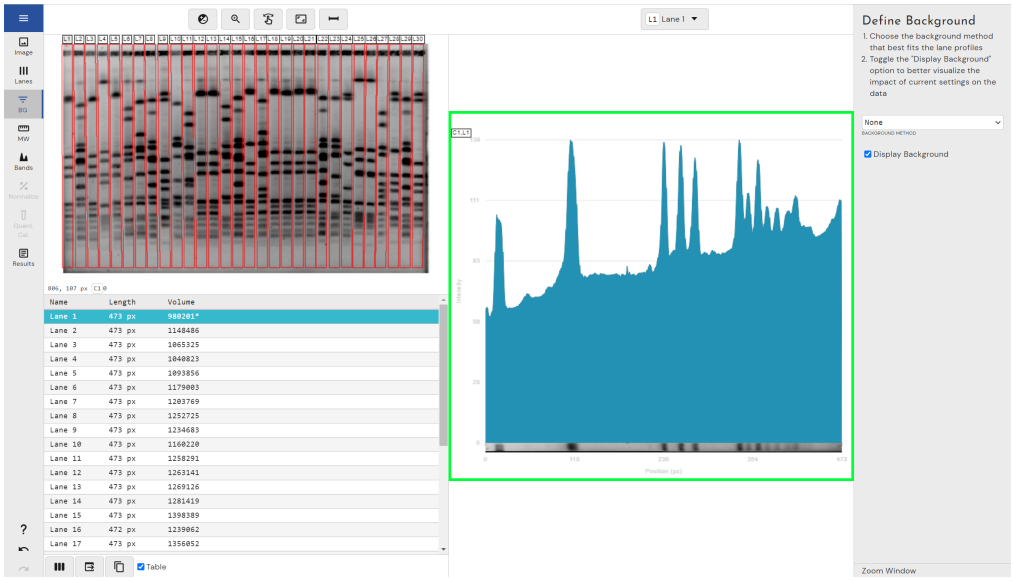
Left-click, hold and drag the diamonds in the center of the lanes to move the whole lane (indicated by the blue box).

To manually resize lanes, left-click, hold and drag the diamond shaped handles at the bottom of the lane box (indicated by the orange box).

Rename

This tool allows you to give lanes specific names, rather than the default "Lane X" which can be useful for labelling samples etc. The names given to lanes will also be matched in the results table and reports exported at the end of the analysis.

Background Mode



This mode allows the user to define the sensitivity and algorithms used for background subtraction.

Background subtraction is the process by which you remove the pixel intensity of the background of your image from your bands of interest to derive their "true" intensity value so that the calculated band volumes represent the volume of the material in the band, rather than the volume of all material including the background.

Please note, this mode is the first time you're introduced to the "profile view" (green) which will be referenced in various areas of this user manual.

Background Subtraction Methods

None

Clears any existing background subtraction.

Rolling Ball

The Rolling Ball method requires you to enter a value for the size of the rolling ball. This method calculates the background as if a disc, with the radius you have entered, were rolling underneath the lane

profile. A smaller ball radius will give a baseline that follows the profile more closely.

The radius should be set in relation to the width of the bands. The rolling ball method will create a baseline that follows the profile of diffuse bands more closely than that of sharp bands.

The radius of the rolling ball is determined as a percentage of the entire lane length. This value can be set:

- Manually – by typing into the Radius box
- Using the slider - between 1 and 100%

The larger the number in the box, the larger the ball so it cannot fall as far into the profile peaks it can fall. This results in a lower pixel value for the background, thus is more sensitive for fainter bands than prominent ones. Using a smaller radius (smaller ball) is more sensitive for fainter bands. This can be visualised by ticking the "display background" box and then changing the ball radius.

This is our recommended background subtraction method because it provides a "moving average" background subtraction which doesn't assume even background values across your lane length.

Rubber Band

The Rubber Band method draws a baseline between the lowest points on the profile, as though a rubber band was stretched under the profile. If the ends of the profile are lower than all other points, the baseline will be a straight line between the ends of the profile, and band separation may be poor. If this is the case, do not use this method.

Constant Value

The Constant Value method allows a user to manually designate a value for the background. This can either be zero, the profile minimum or by selecting a value on the profile itself by left-clicking

This subtraction method is not recommended as it assumes the background to be constant across the entire gel/blot which is almost never the case.

Profile min.

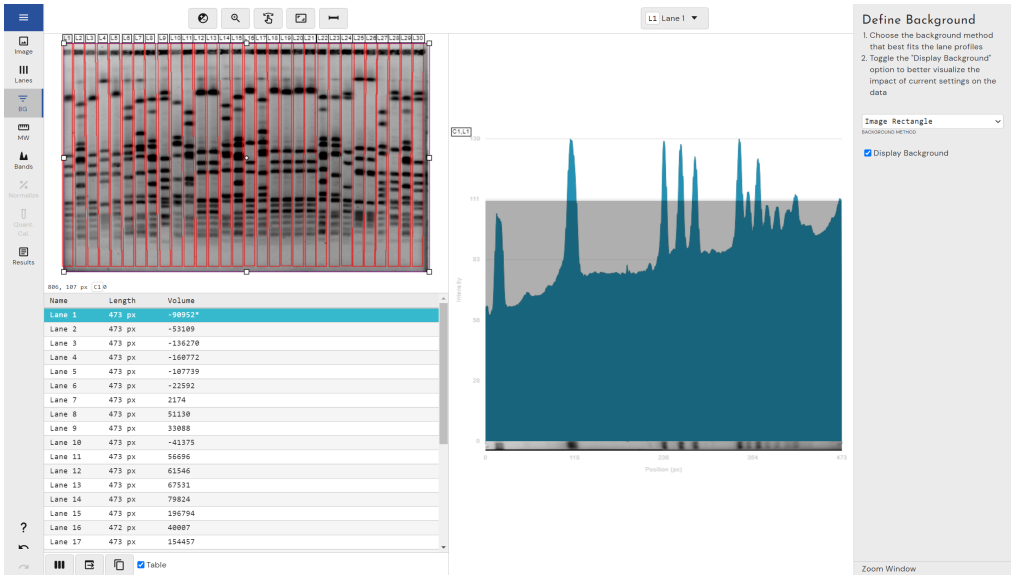
This method sets the background level as the lowest value found in the profile. It is not advisable to use this method when the lowest

point is found at the extremities of the lane since the result is dependent on where the lane is drawn. This calculation can be hard to repeat between analyses.

Image Rectangle

The average intensity within a rectangular area on the image is taken as the background intensity.

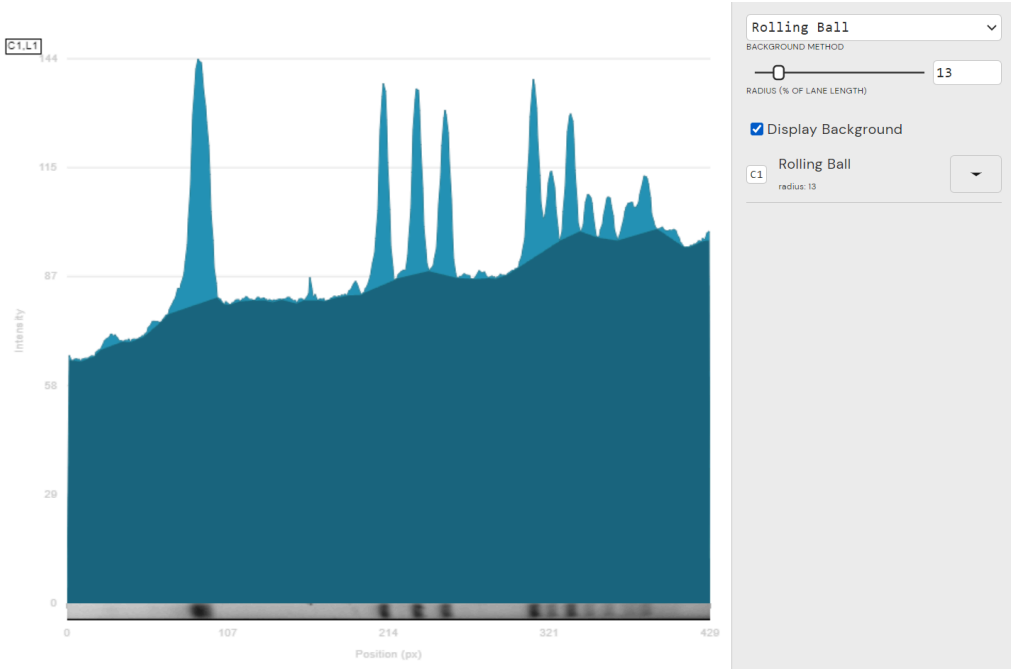
On selecting image rectangle from the drop-down list, a user editable rectangle appears in the image view to the top left of the display:



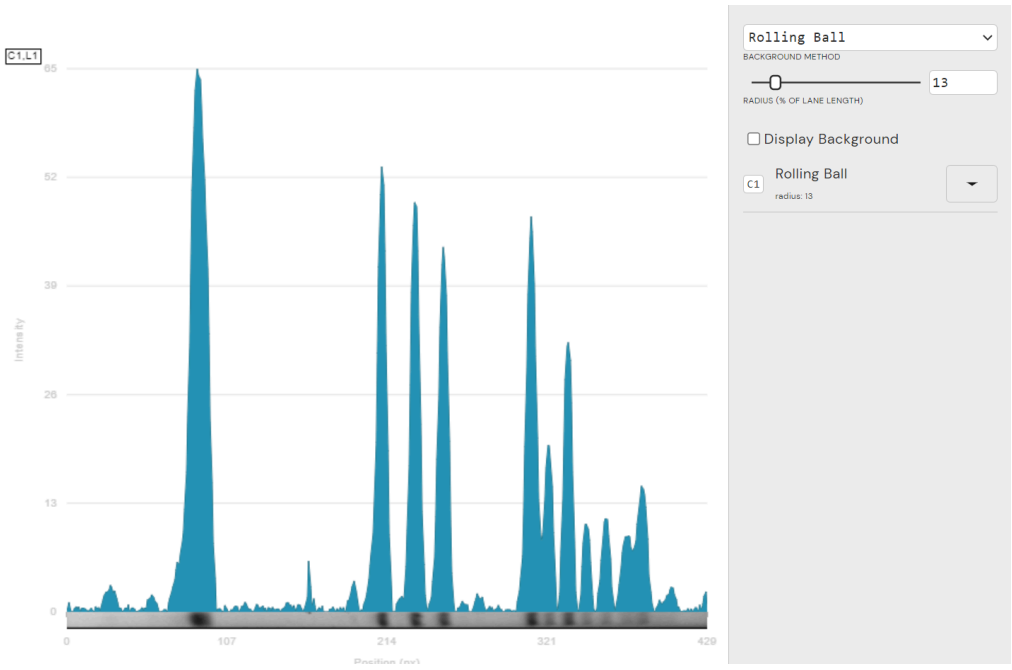
Use the handles on the edges of the rectangle to resize it and the diamond handle in the center to move it. From this rectangle, the background intensity is applied and calculated for all lanes.

Display background

This tickbox turns on/off the background display in the profile view to help visualise how the current background removal settings will impact peak height and volume.



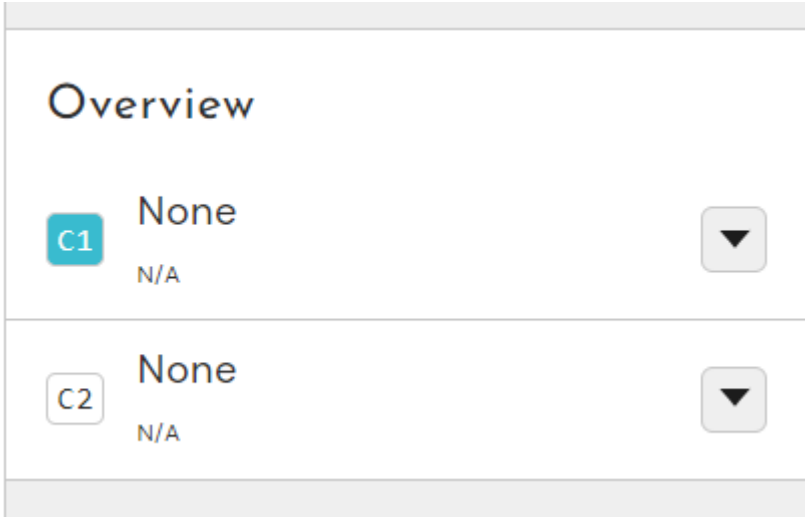
Display background checkbox ticked, background that will be removed shown in dark blue



Display background checkbox unticked, lane profile is shown with background subtracted based on current background subtraction settings.

Channel Selection

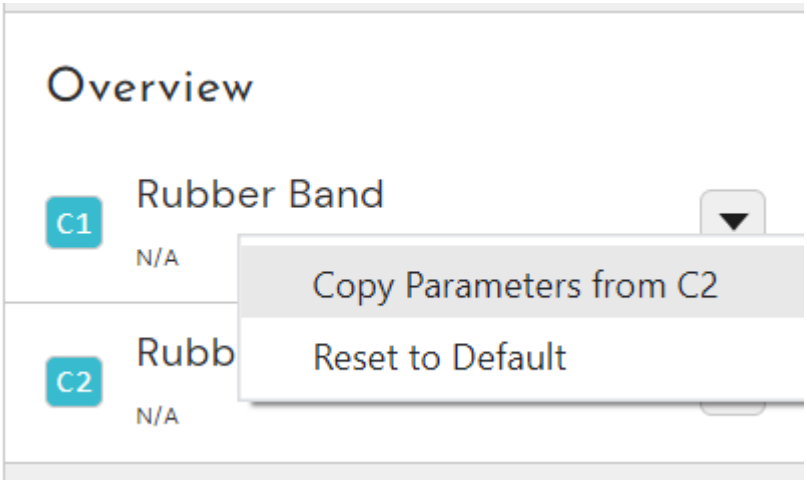
This option is only available when using a multichannel image for analysis.



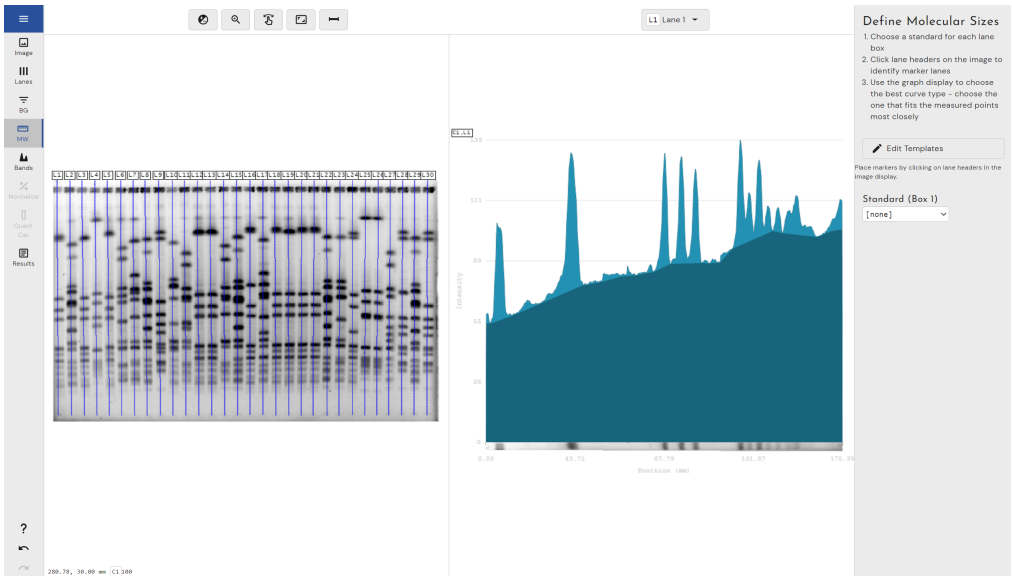
When using a multichannel image, each channel of the image can have it's own seperate background subtraction method and settings.

Background subtraction methods are applied to whichever channel (or channels) are currently selected (ticked) in the top taskbar.

Background subtraction methods and settings can be copied between channels by left-clicking on the arrow to the right of the channel name and selecting "Copy Parameters" on the lane you want to copy the parameters to.



MW Mode



The Molecular Weight Calibration Window uses a standard lane within your lane box to determine the molecular weight of all the bands across the whole lane box.

The correct MW marker can either be selected from several industry standard options or added manually and then calibrated using a number of curve types. If more than one Lane Box has been drawn, a different marker can be applied to each one.

> *Please note, whilst this mode is referred to as molecular size calibration, it can be equally be used to determine pI values on isoelectric focusing gels or migration properties on TLC plates.*

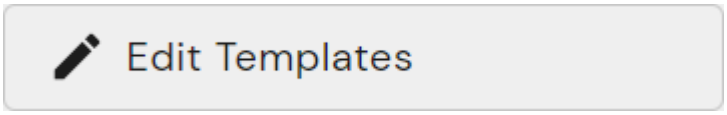
For reference, the calculation for MW of samples within Phoretix 1D is fully compliant with FDA Q4B Annex 10(R1): Polyacrylamide Gel Electrophoresis General Chapter Guidance for Industry and the guidance set out in the European, Japanese and United States pharmacopeias.

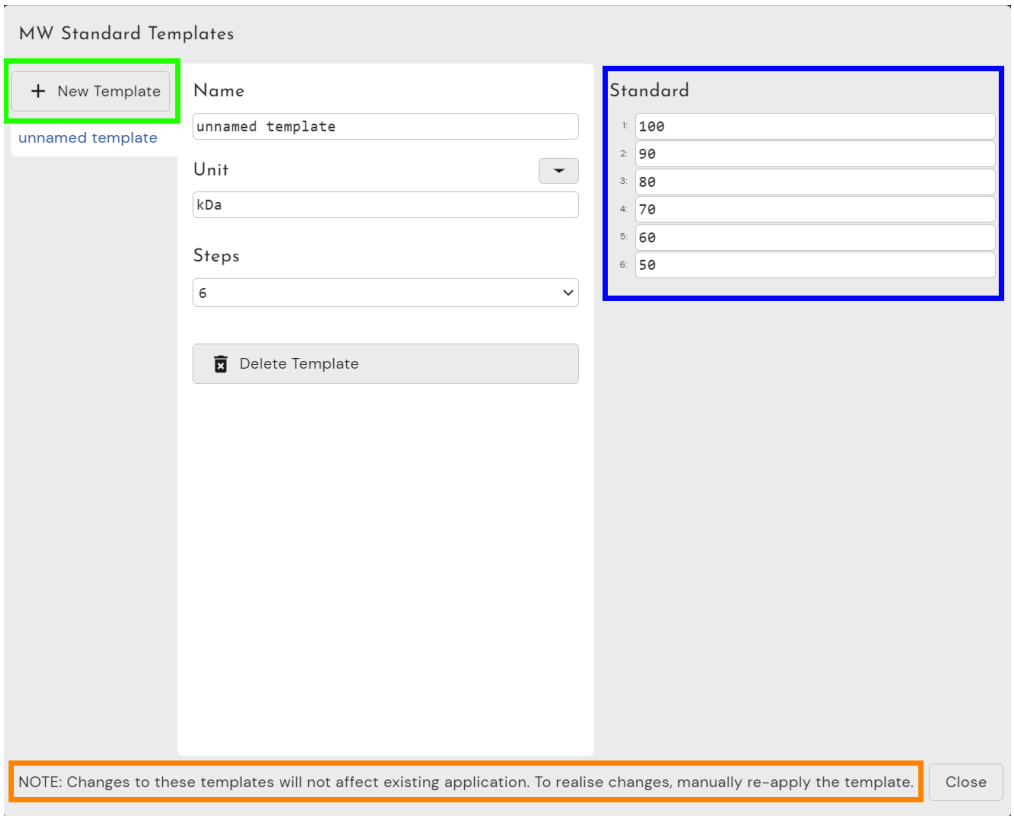
Briefly, the migration distance of the unknown samples from the top of the lane is divided by the total lane length (presuming the bottom of the lane is aligned with the bromophenol blue dye front) to obtain the unknown samples relative mobility (Rf). The RF values for the known MW standards are plotted and the RF values for the unknown samples can be converted into estimated MW values by linear regression analysis against that graph.

This is how Phoretix 1D calculates MW for unknown samples.

Edit Templates

Click this button to open the template editor and create your own MW standard templates:





To create and add a new template, left-click on "New Template" (green box).

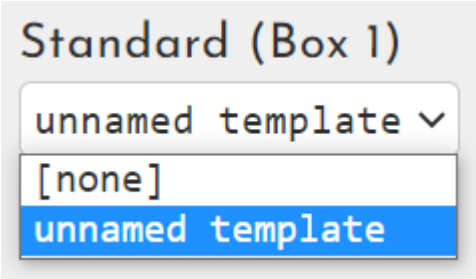
Then enter the name of the template, the units of the MW standard (either by typing it in the box or selecting from the drop down arrow) and the number of steps (bands) in the MW ladder.

Once you've defined the number of steps in the MW standard, assign each step its known value from largest to smallest (blue box).

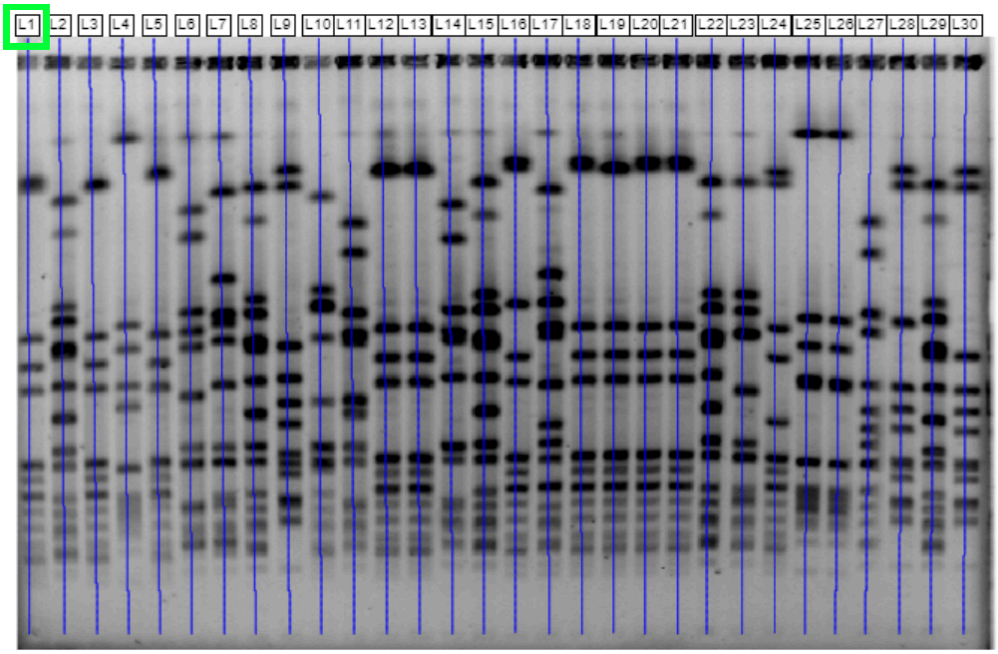
> *It's important to note that any changes to MW standards will not be automatically applied and will require you to re-apply the standard to the standard lane (orange box)*

Left-click "Close" to exit the MW standards template editor and save your changes.

To apply your MW template to your image, first select it from the drop-down menu:

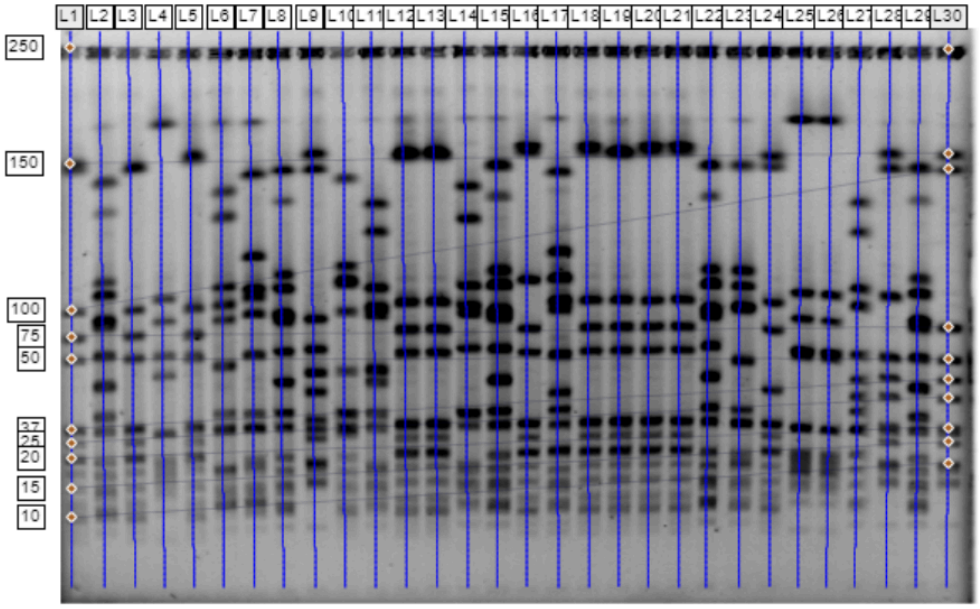


and then click on the name of the lane label of your standard lane (typically this will be L1):



You will see your MW standard steps are automatically assigned to the detected bands in those lanes.

If you have multiple MW standard lanes within the same lane box (for example, one at either end or one in the centre) simply left-click on those lane labels as well to add the same MW standard to those lanes.



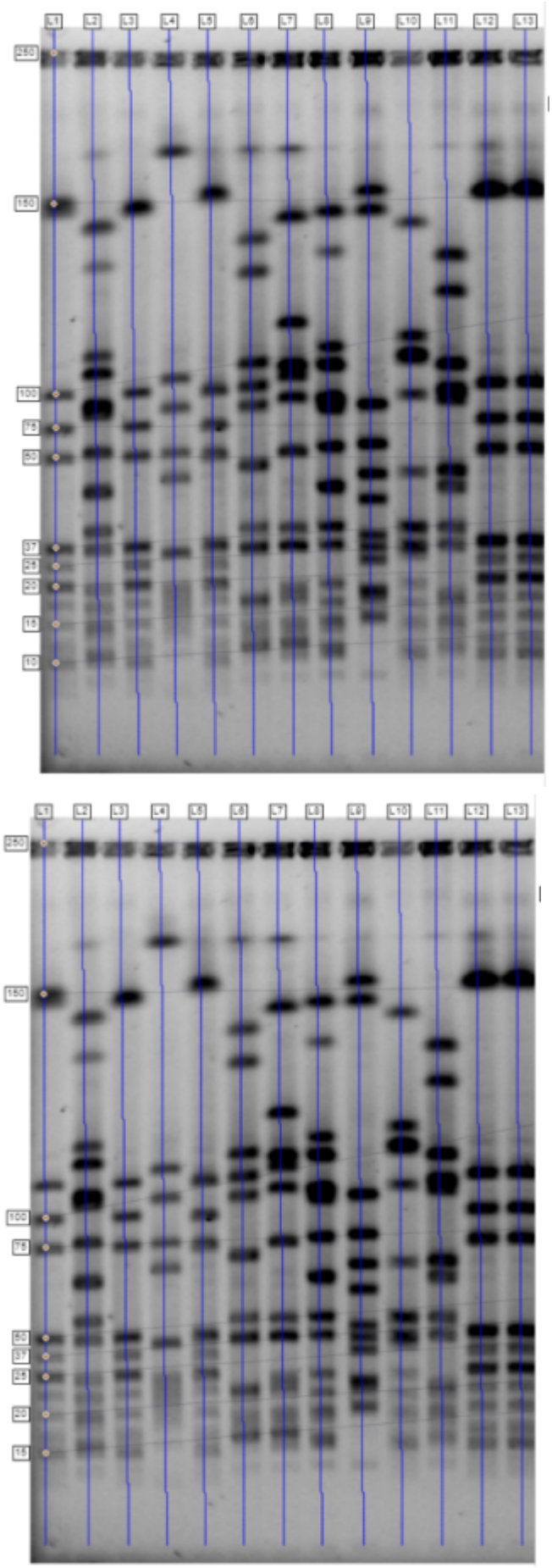
As you can see, when multiple MW standards are present on an image they attempt to join across the image. These are used as reference points for MW calculation and any distortions in the gel can be accounted for mathematically by taking into account how the standards have moved at either end.

We would always recommend planning your experiment to contain MW standards at either end for more accurate MW calculation sizing. For increased accuracy you could also consider a MW standard lane in the center of your gel.

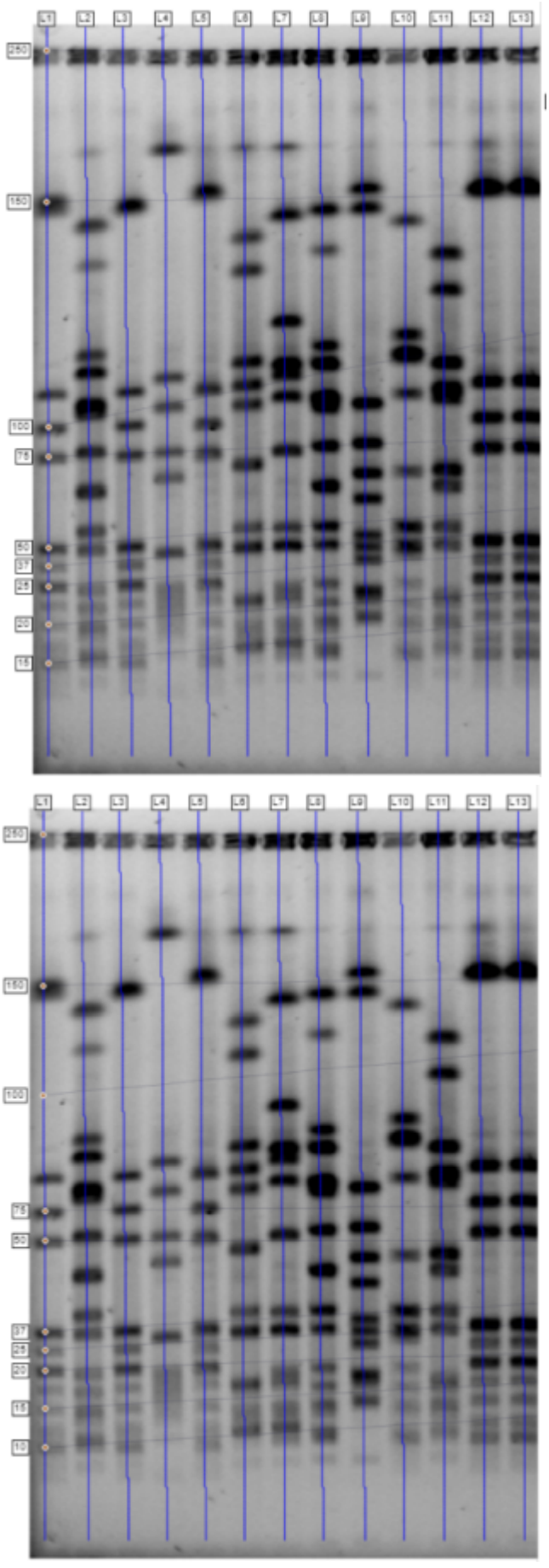
If you wish to remove a MW ladder, either right-click on the lane label at the top of the lane or left-click the "X" button to the right of the lane number on the right hand side taskbar.

Editing MW Steps

If the bands are incorrectly assigned a value simply right-click the red square on the band in the image view to remove that band from your MW standard:



If you wish to add a band to your MW standard lane, simply left-click on the area where you would like the band to appear:



You will notice that the addition/removal of bands in the MW standard automatically relabels all the other bands to align with the number of steps expected in the MW standard.

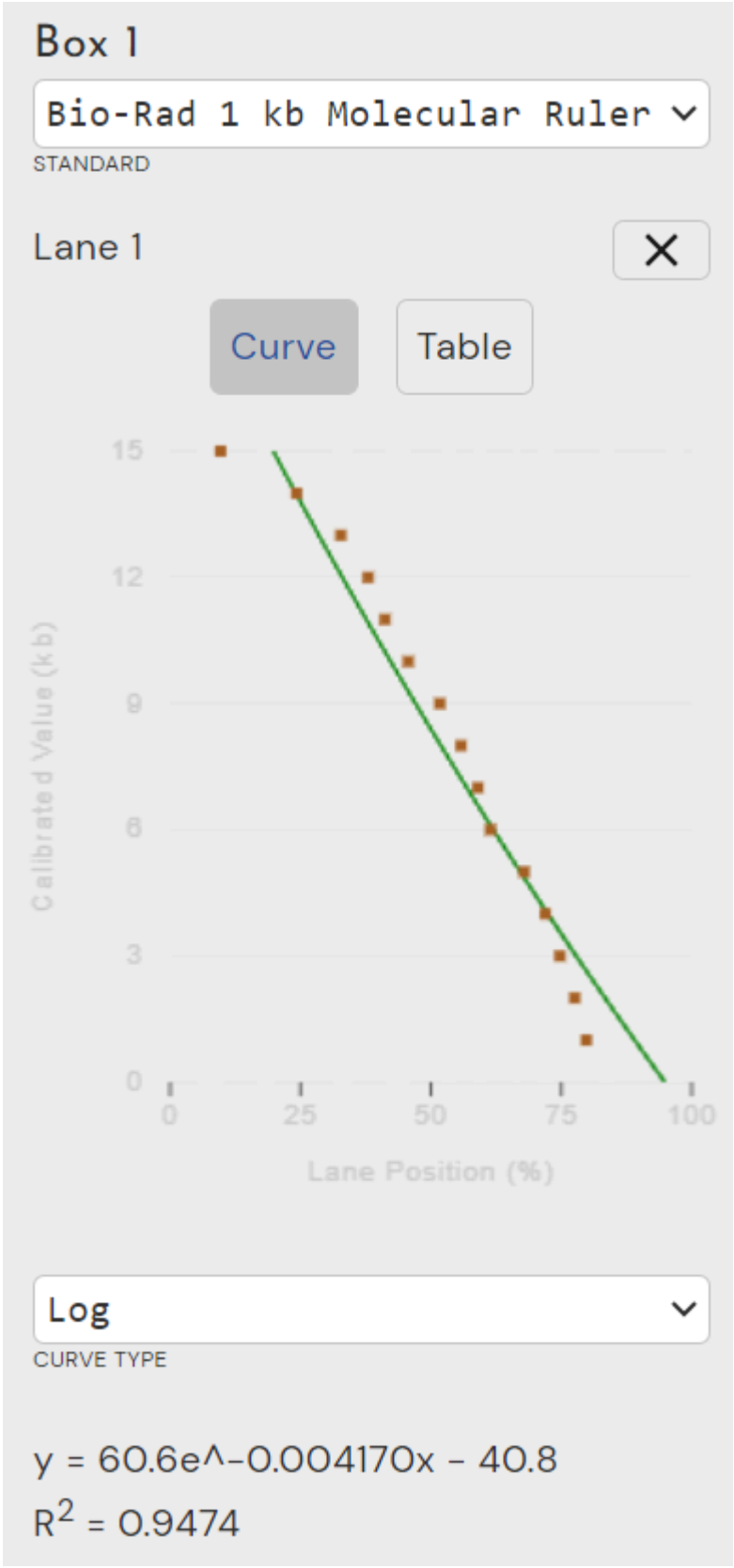
Built-in MW Ladders

Phoretix 1D comes pre-bundled with a number of very popular MW ladders which can be selected from the same dropdown menu as your custom templates and applied in the same manner.

This is in no way an endorsement of any particular MW standard and standards are liable to be changed by the manufacturer at any time. Please make sure if using one of the pre-bundled ladders that the values for each MW step match those in the Manufacturer's documentation for that batch.

MW Equations

Once a MW ladder has been applied to an image, the equation used to calculate values between MW steps is displayed alongside a graph of values vs MW markers:



Where the orange diamonds are your known MW values (i.e. the steps in your MW marker) and the green line is the calculated values according to the selected equation.

The line fitting equations used in Phoretix 1D are:

> *Log: y = aln(bx)*
> *Straight Line: y = a+bx*

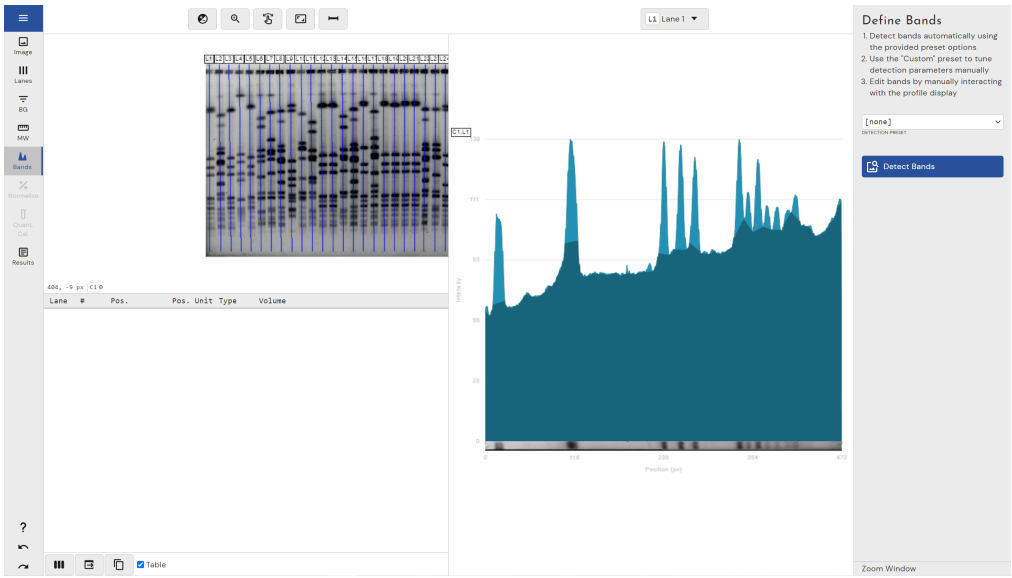
> *Power: y = ax^b*
> *Exponential: y = aexp(bx)*
> *Exponential (offset): y = aexp(-bx) + c*

Please note as First-order Lagrange and Cubic Spline are not equation-based methods of line fitting they don't have an equation associated with them. These methods simply join the known points.

It's recommended you use the fitting algorithm suggested by the manfucaturer of your particular MW ladder. If you're using in-house derived ladders or the manufacturer doesn't supply such information please use the line-fitting algorithm that gives an R square value as close to 1 as possible. The closer the R squared value is to 1 the more accurately the extrapolated values align with the known values and the more confident you can be in their accuracy.

These equations are the same ones used in the Quantity Calibration and Calibration (21 CFR/GxP version only) workflows.

Bands Mode



The Bands mode offers you automatic methods for detecting bands and also allows you to edit those bands manually after detection.

There are two main parameters governing band detection: the detection of band peaks and the detection of band edges.

The peak of a given band is the point in its profile where the image intensity is at its maximum value. This is used to define the band's

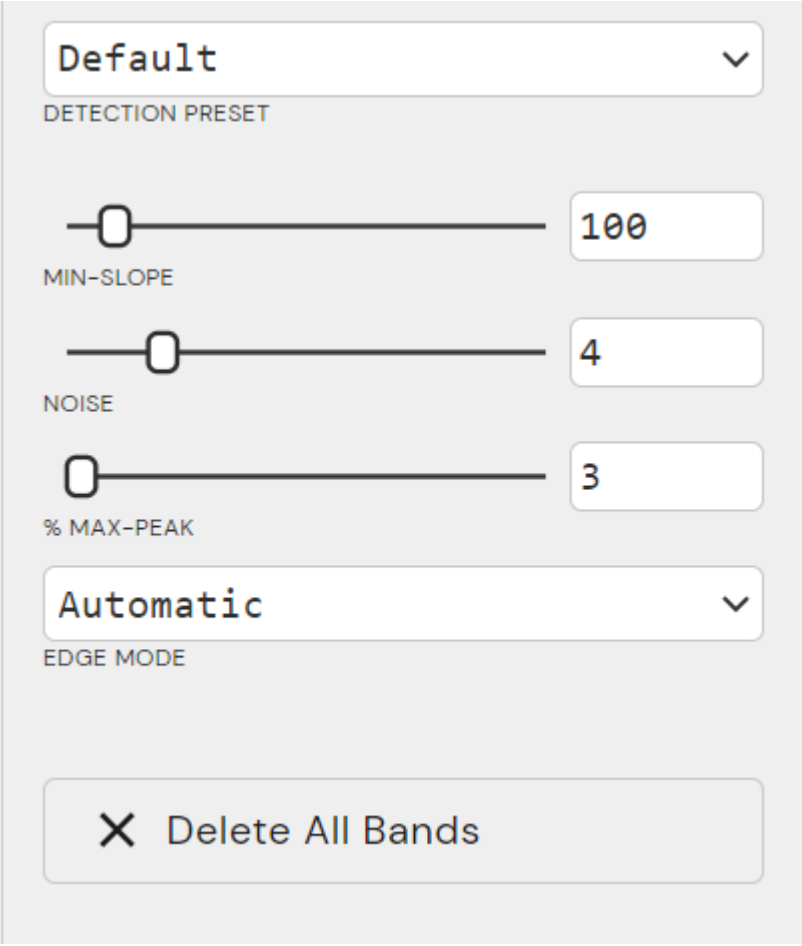
position in the lane. The bands are rarely a single pixel in length; therefore, the extents of the band must also be determined so you can measure the band's volume.

Automatic Band Detection

Automatic band detection uses a series of algorithms to find the peaks in the profile to declare as bands and the troughs between them to declare as edges.

Automatic Band Detection Settings

There are some parameters on the right-hand side of the window which can be manually defined by the user to influence the algorithms sensitivity to peak/edge detection if necessary:



To access these, select the "default" option from the dropdown menu on the right-hand side.

Min-slope

This parameter defines how pronounced the band must be from its surrounding area in the lane. The range for this parameter is 0–999.

A high value favours only detecting bands with a steep gradient whilst a lower value allows the gradient to be less severe.

In general, the lower the minimum slope value, the more bands are detected.

Noise

This parameter represents the degree to which small local peaks should be ignored and is designed to eliminate noise in images. Noise reduction has no effect on the profile itself, only the number of peaks detected. The range for this parameter is 0–20.

Typically, the higher the noise reduction value, the fewer peaks detected.

% Max-Peak

This is a threshold parameter, which discards peaks under a certain size in relation to the most intense peak on the gel. The higher the percentage value entered here the fewer the peaks likely to be detected in the profile. The sizes of the peaks are calculated after background subtraction. The range for this parameter is 0 - 100.

In the Advanced options dialog you can set this parameter to work from the highest peak in each lane rather than the whole gel

Edge mode

Single Edge

This option will automatically add a single edge, between the bands, at the minimum profile value. It will also stop you having more than one edge between bands, when editing edges. This essentially creates bands to cover your entire lane length.

Automatic detection

Select this parameter if you want the software to automatically detect the band edges. Automatic detection is the preferred method for edge detection. The software identifies an edge as the trough in the profile on either side of the band's peak.

Fixed width

If you want to specify the width of each band (in pixels) thereby determining the position of each edge, select the Fixed Width

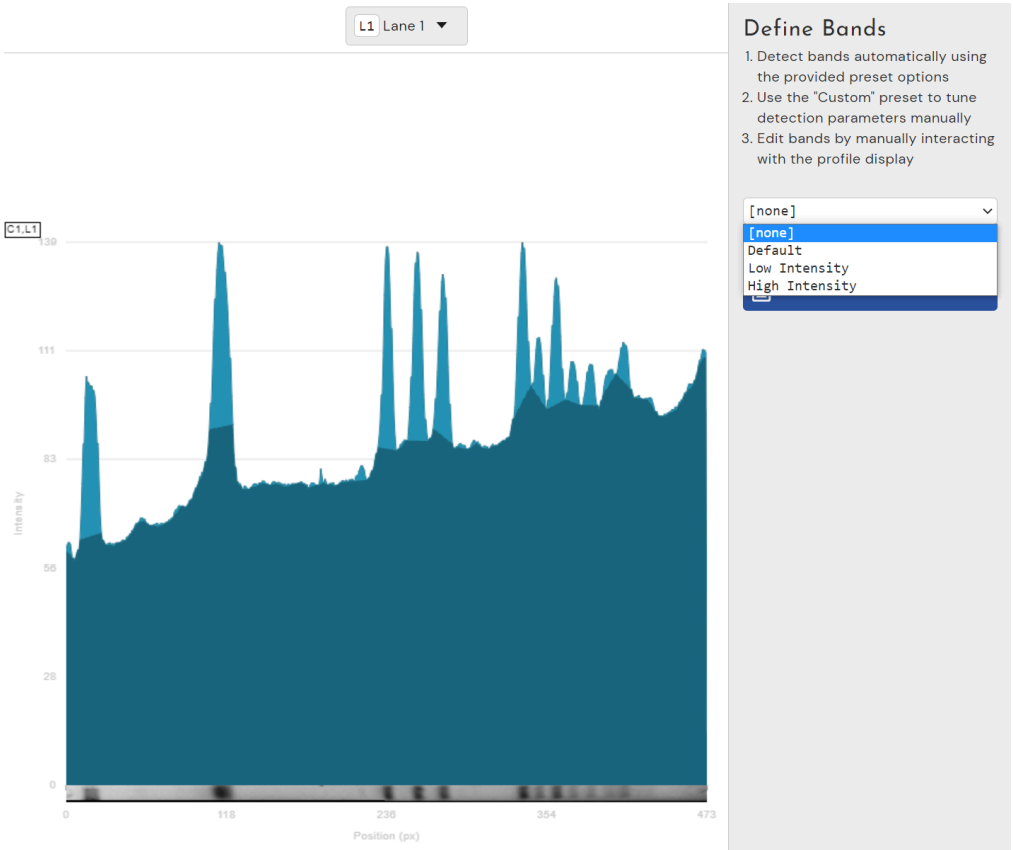
button and enter the required width. The band edges are positioned at an equal distance on either side of the peak.

Percentage of peak

The system reads the intensity at the peak and then moves the edges outwards, until the difference between the intensity at the peak and the intensity at the edge is equal to the specified percentage. For example, if peak intensity were 100 and your percentage of peak were set to 5%, your edges would move outward until they hit an intensity value of 95 and be placed there.

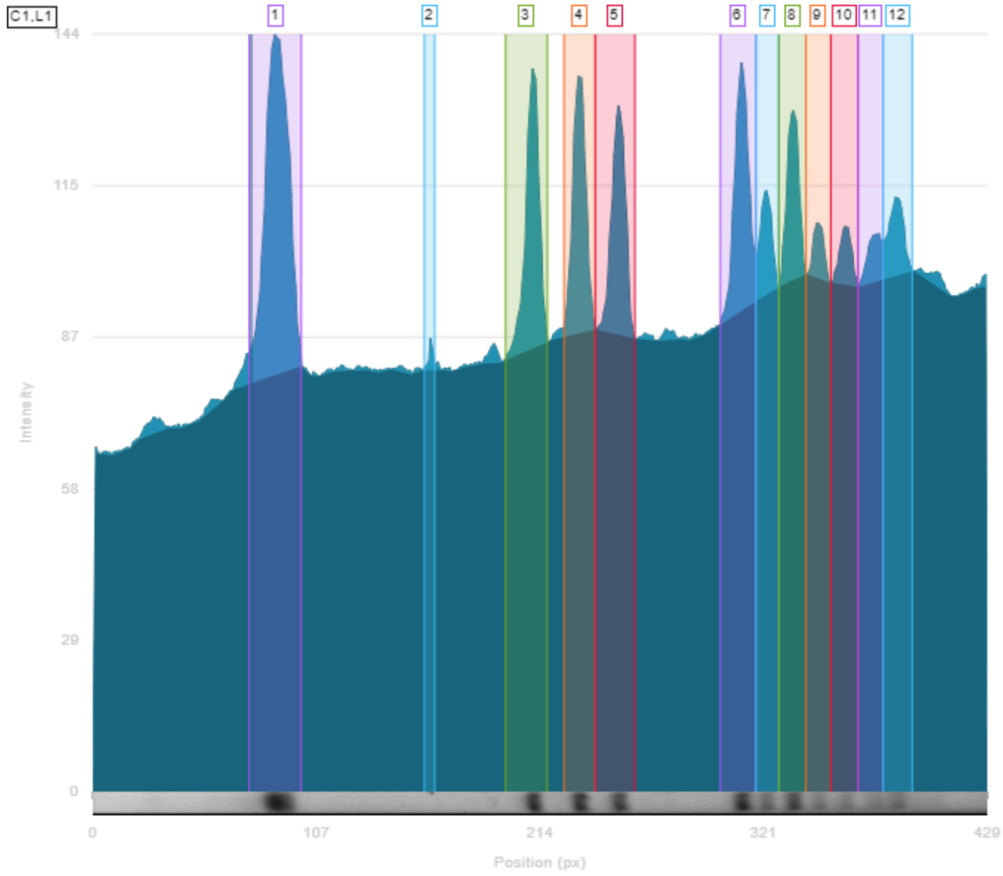
Prebuilt automatic band detection settings

There are a number of built-in default settings for the automatic band detection algorithm which can be selected from a dropdown menu on the right hand side of the window:



"Low Intensity" is designed to detect fainter bands for analysis
"High Intensity" is designed to detect more intense bands for analysis

Manually editing bands



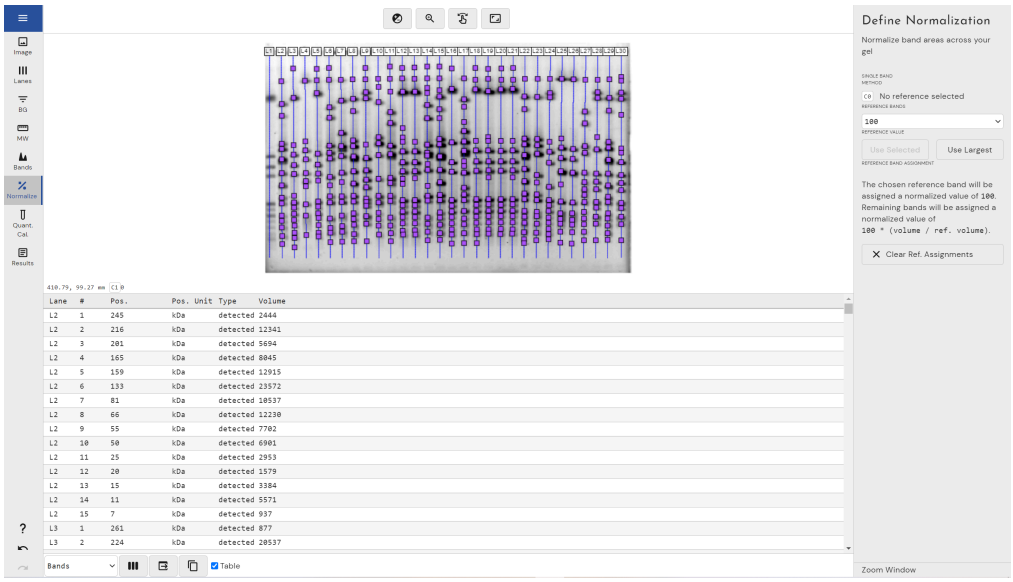
To manually edit the edges of existing bands, left-click, hold and drag the edges of the band to where you want them to be.

If you wish to split an existing band, right-click on the band and select "split here" from the menu.

To delete individual bands, right-click on the band in the profile view and select "delete band" from the menu. You can also right-click on the band in the image view to delete it.

To add a new band, left-click on the profile view where you want the band to be placed, hold the left mouse button and drag to where you want the band to end before releasing.

Normalisation Mode



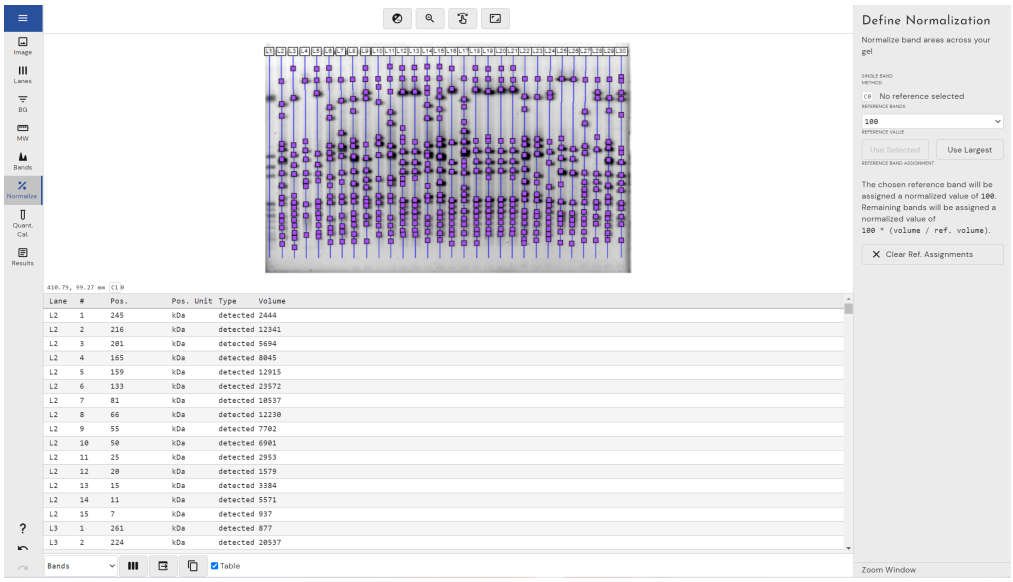
The Normalisation mode is used to generate the value of normalised volumes for the bands in a gel. This entails:

• Setting the normalised volume for specific band in a lane if a standard gel.
• Selecting a channel in a multiplex gel which contains housekeeping proteins/genes
• Selecting a channel in a multiplex gel which uses total protein in each lane to normalise to.

It then recalculates all normalised volumes for the other bands in the gel using the normalisation factor.

Performing Normalisation for a Standard (single-channel) Gel

The only option available for normalising a single channel gel is single band normalisation:

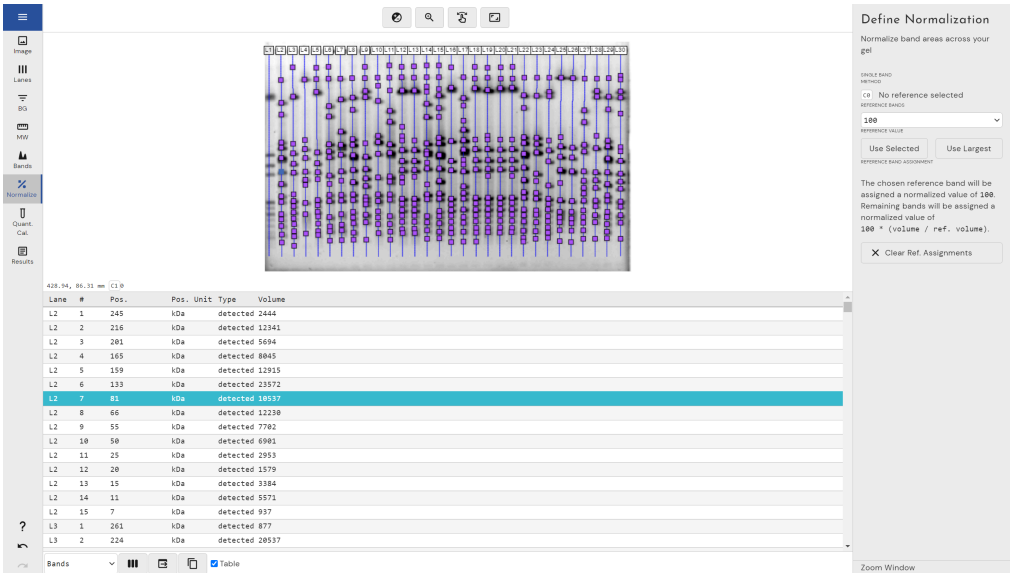


Single band normalisation uses the volume of a user selected band and sets this value to 1,10,100,1000,10000 or 100000:

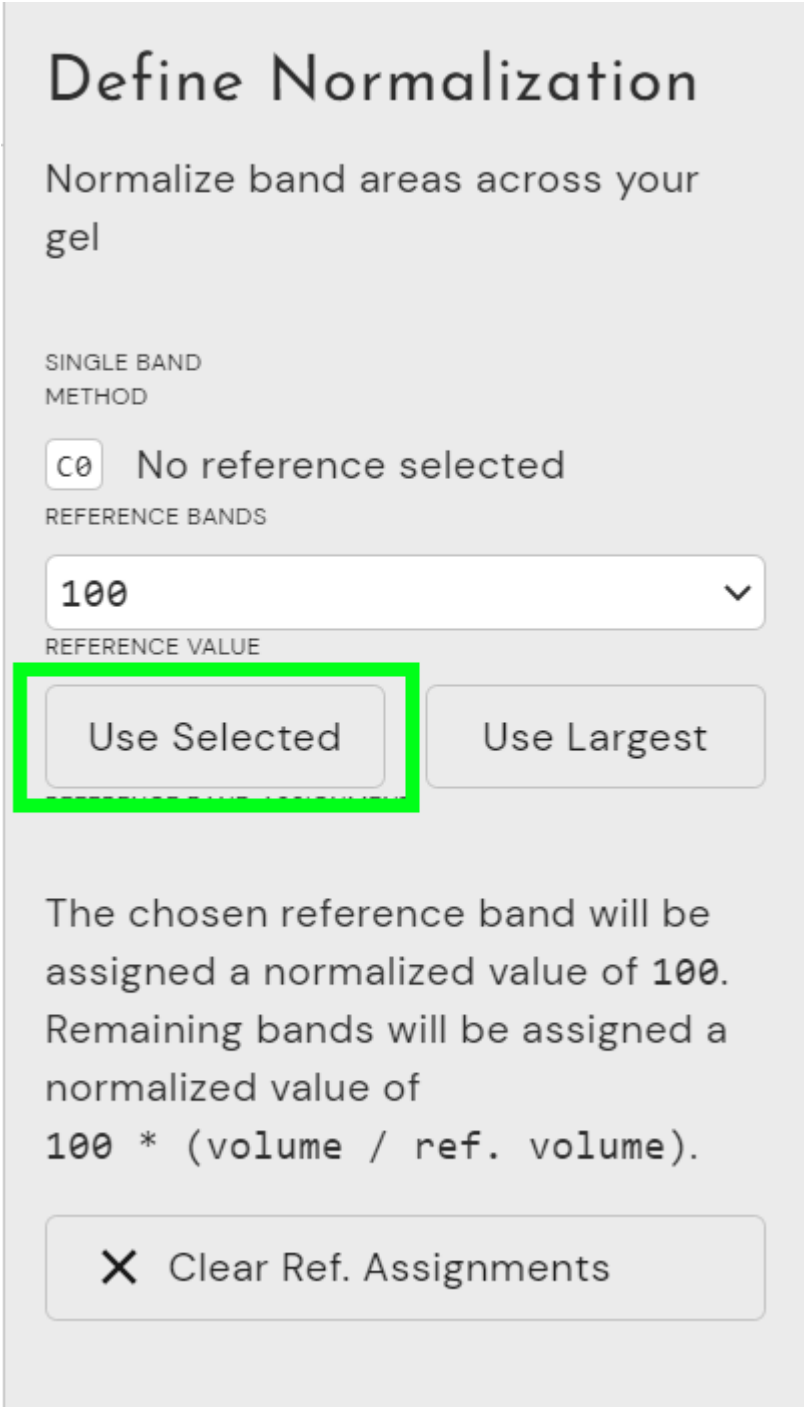
It then uses this formula to calculate the relative abundance in each band on the gel relative to the one you selected:

> *100 x (volume of band/volume of reference band).*

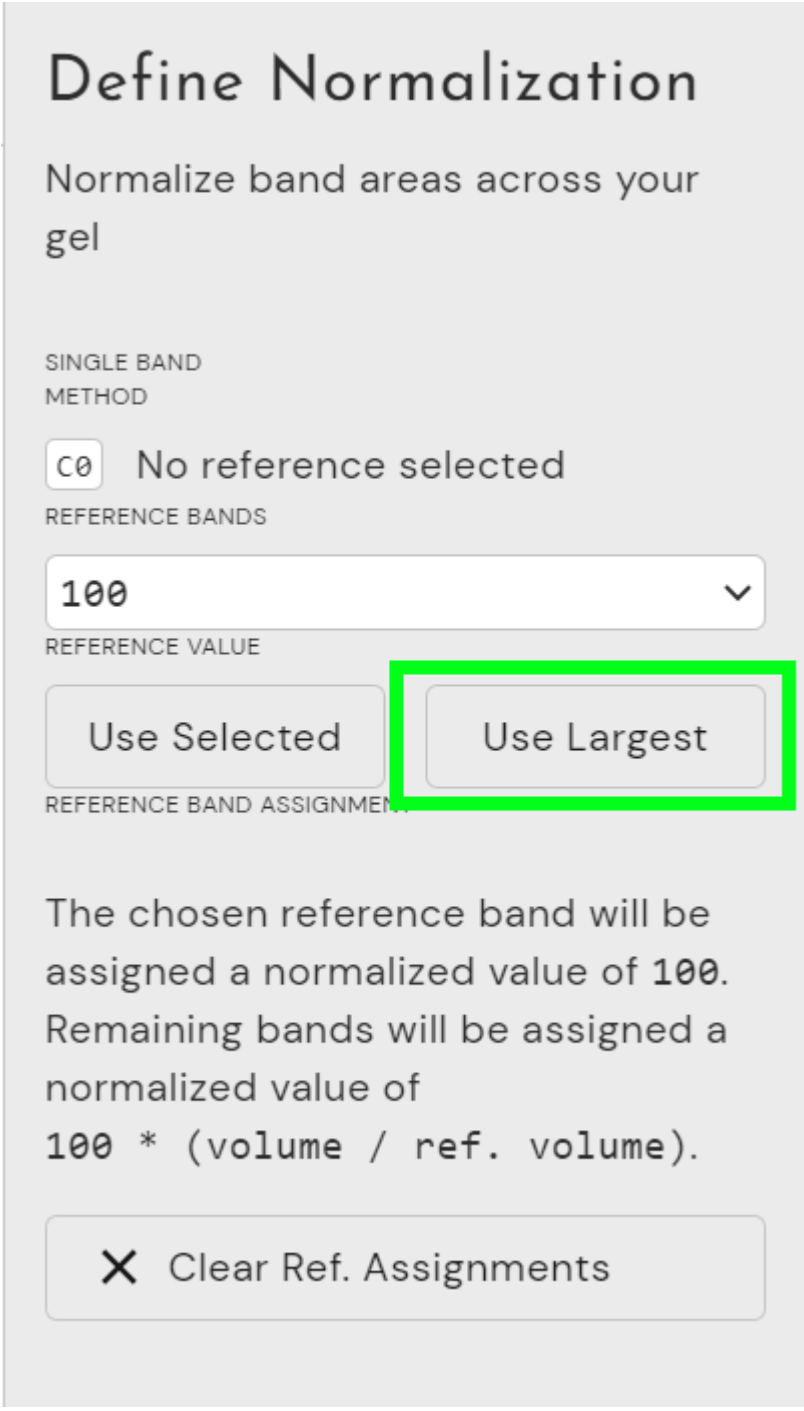
To select your reference band, left-click the band on either the image view or in the results table:



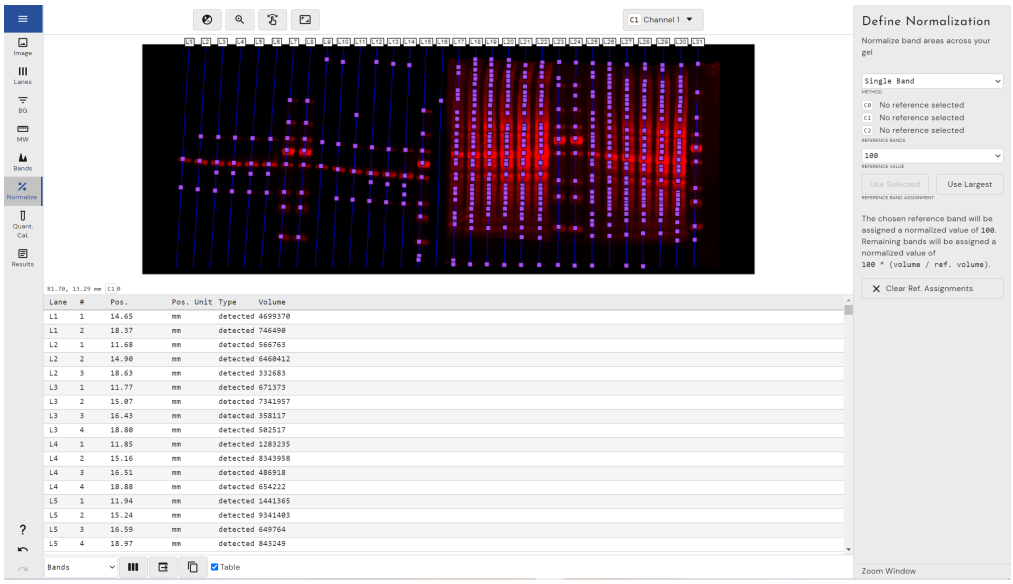
Then left-click "Use selected" on the right-hand side:



If you wish to automatically detect the most intense band on the entire gel and use that for your reference band, select "Use Largest" instead:



Performing Normalisation for a Multiplex Gel



Alongside being able to normalise to a single band as you can on single channel images, multiplex images also have the options to be normalised to total lane volume or housekeeping proteins/genes.

Normalising to Total Lane Volume

Normalising to total lane volume uses the total protein content of the lanes in your reference channel to compare to all the lanes in your other channels.

This option is used if you have one channel of your gel assigned with lanes that contain a defined total protein.

The idea is that the volume of all the material in each lane should be identical (i.e. the volume of all the peaks should add up to represent the same total protein) however, as it's likely they will all have slightly different values, a normalisation factor is calculated for each lane to accommodate for this variation.

Phoretix 1D will automatically detect the most abundant lane within your selected reference channel, then apply the following normalisation equation to every other lane in the reference channel to normalise them:

> *NF = volume of all protein in each lane/volume of total protein in the most abundant lane*

Now, once all of the total protein lanes have been normalised, normalisation for the bands in the other channels is performed using the following equation:

> *Normalised band volume = raw band volume x NF*

Normalising to Housekeeping Protein

Normalising to housekeeping protein/gene is used when you have one channel of your gel assigned with housekeeping proteins or genes.

The largest band by volume (i.e. the most intense band) *in each lane* in the selected reference channel is automatically identified and marked as the housekeeping band (HK) for that lane. The most intense band *in the entire gel* for the reference channel is then automatically detected and identified as the reference band (RB).

With this information, each lane in the reference channel is given a normalisation factor (NF) by comparing it to the lane containing the reference band using the following equation:

> *NF = volume of RB / volume of HK band in each lane*

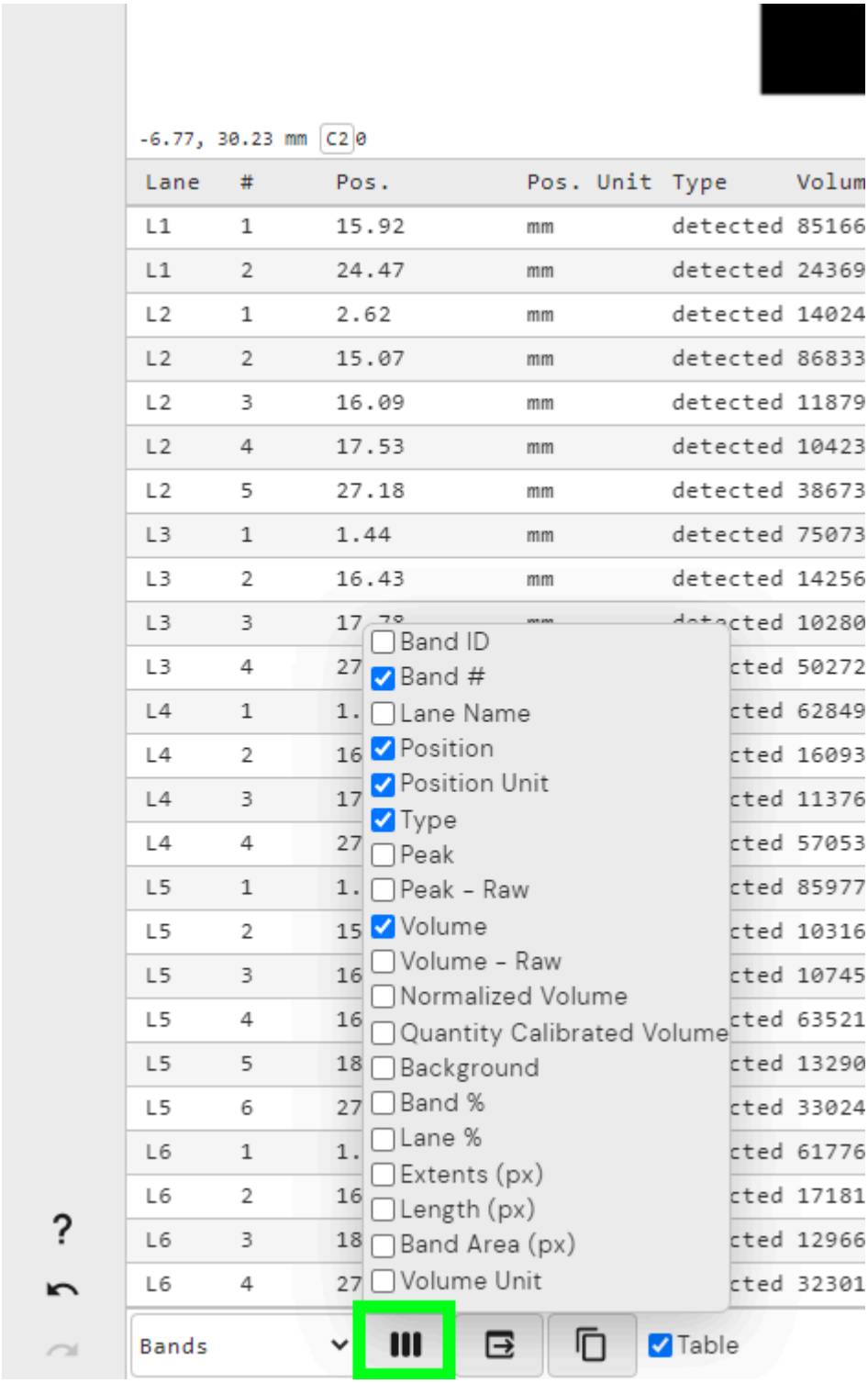
Now, once all the housekeeping bands have been normalised to each other in the reference channel, normalisation for the bands in the other channels is performed using the following equation:

> *Normalised band volume = raw band volume x NF*

Displaying normalised values

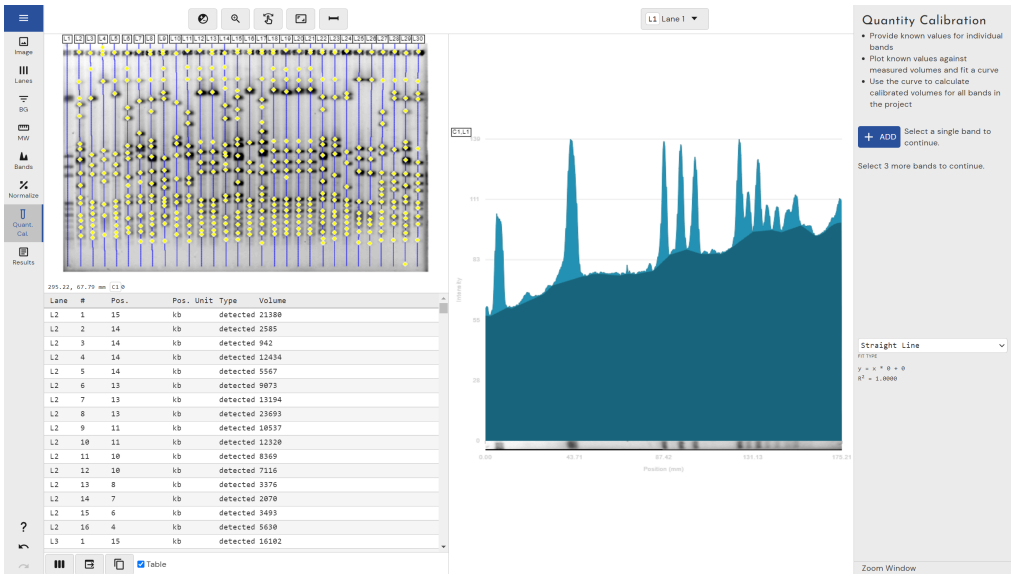
Normalised values can be displayed in the results section of the window in one of two ways, either in the main results table or in a dedicated normalisation table.

To turn on the normalised volume in the main results table, left-click the results table menu:



To access the view options and left-click the "Normalised Volume" checkbox.

Quantity Calibration Mode

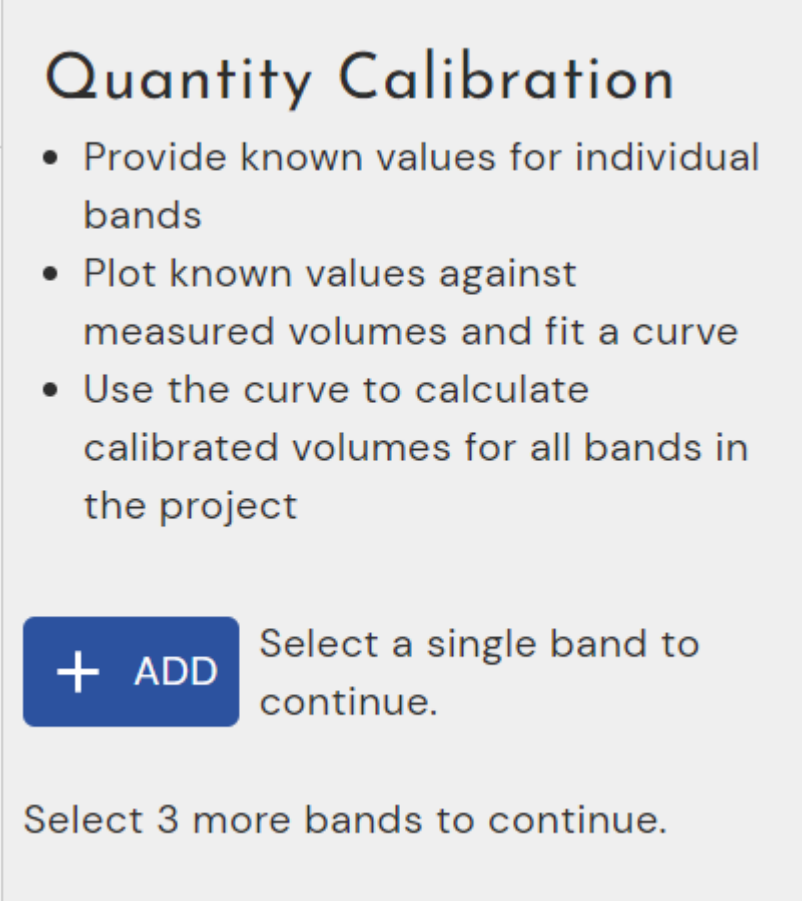


The quantity calibration mode is used to convert detected band volumes to real-world values by including known value standards in your gel/blot experiment and then defining those values within Phoretix 1D.

Phoretix 1D requires a minimum of 3 bands with known values to generate a standard curve with which to fit all band values to.

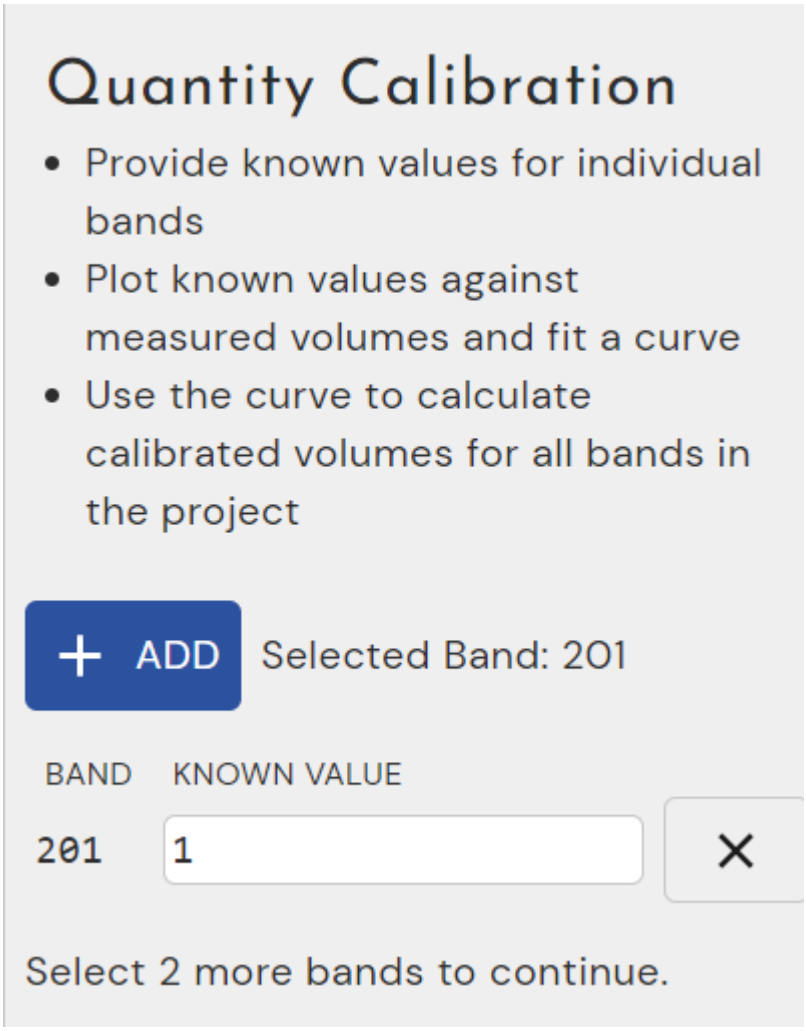
Selecting and adding bands of known quantity

To select your known quantity standard bands, you may either left-click on them in the image view or in the results table. Once selected, click "add" on the right-hand side taskbar to add that band's volume to your calibration curve:



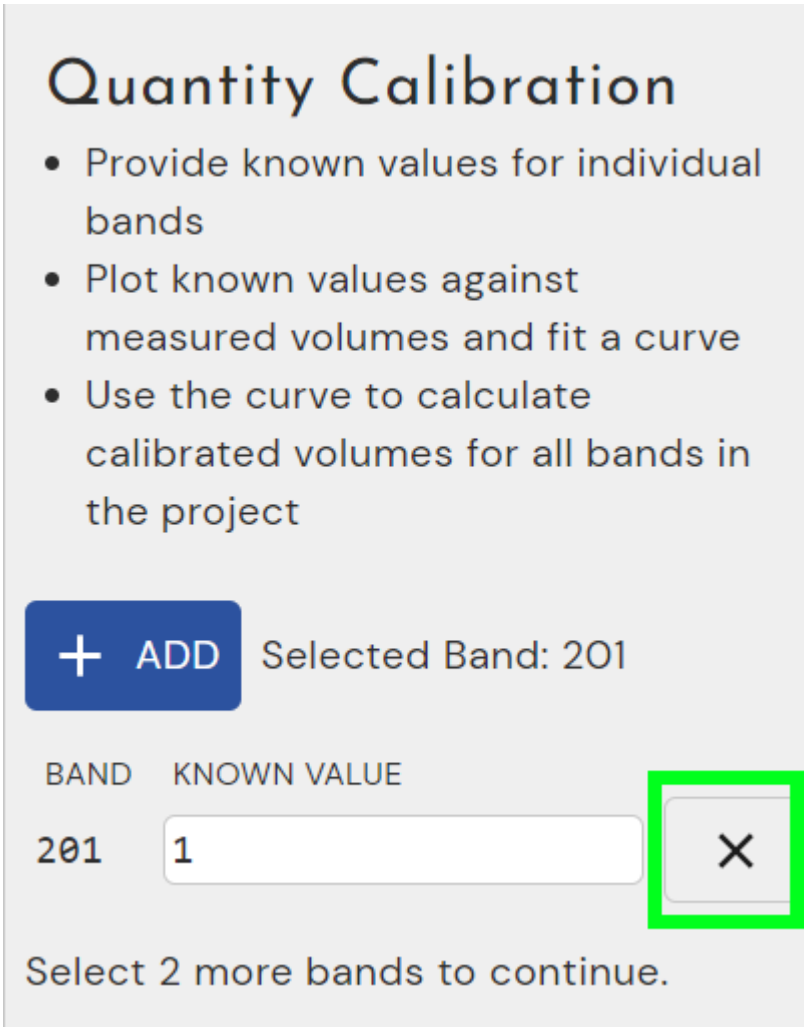
Assigning known band quantities

Once your known quantity band has been added as a data point to your calibration curve, you are able to manually assign it a value:



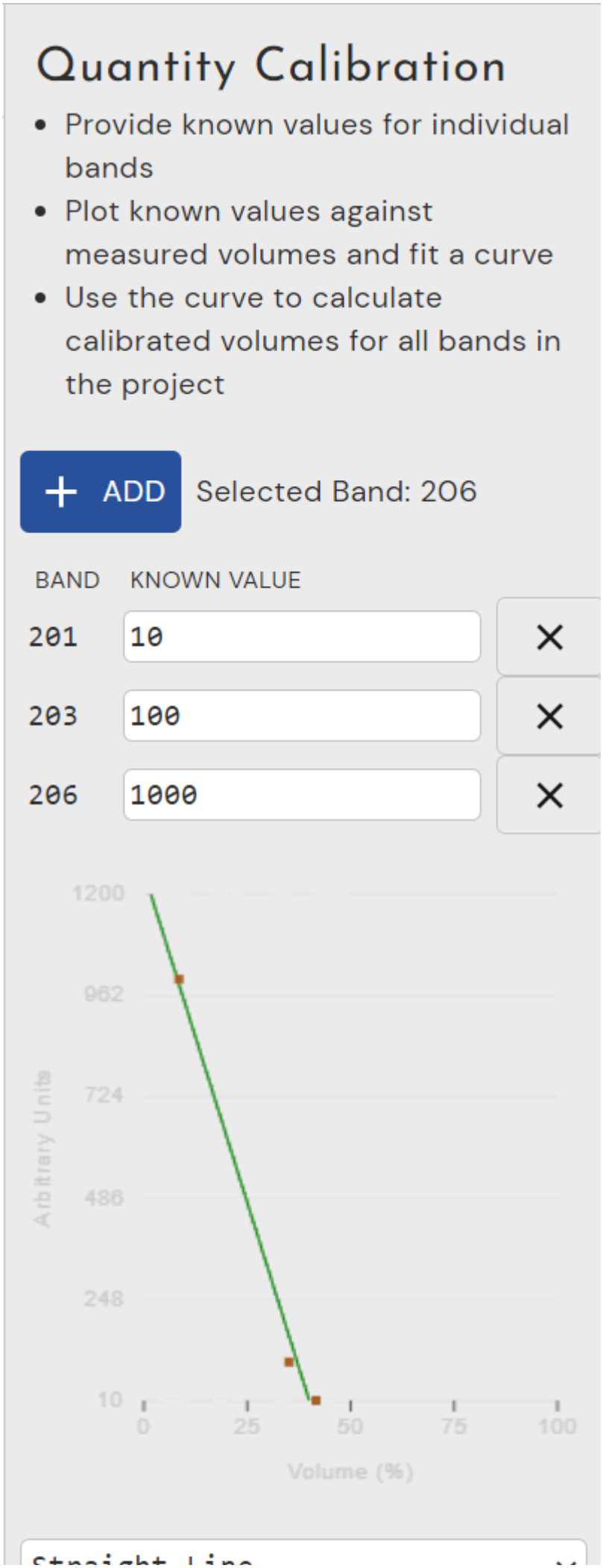
These values are intentionally unitless to give you maximum flexibility within your experiment, be it picograms of protein or number of base pairs in a DNA samples.

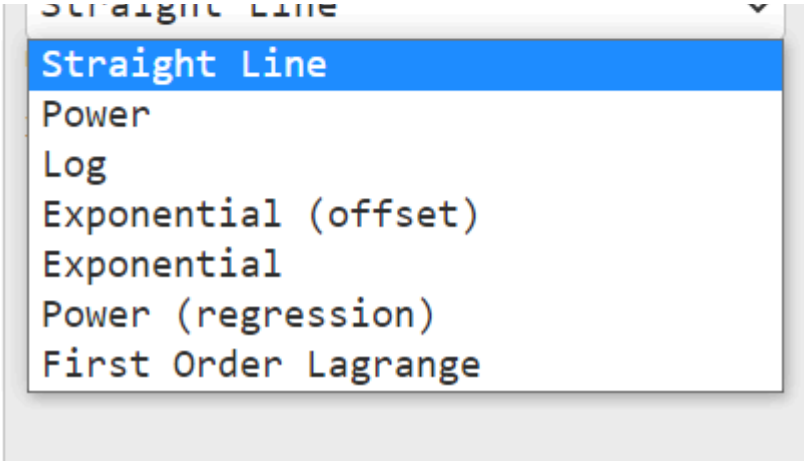
To remove a band from your calibration curve, simply left-click the "X" button to the right of the known value input box:



Designing your calibration curve

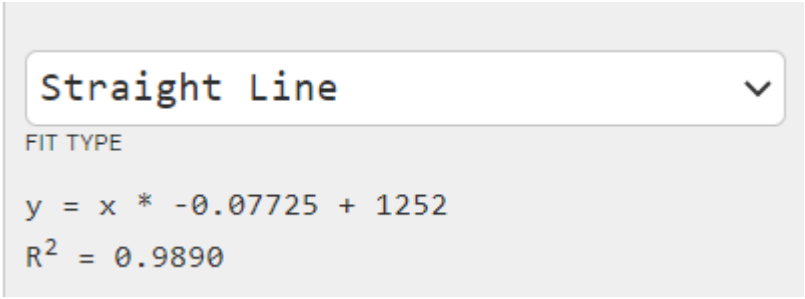
Once you have added at least 3 bands with known values you will be able to view your calibration curve in the right-hand side taskbar:


Quantity Calibration
Provide known values for individual bands
Plot known values against measured volumes and fit a curve
Use the curve to calculate calibrated volumes for all bands in the project
ADD
Selected Band: 206
BAND
KNOWN VALUE
201
10
203
100
206
1000
1200
962
724
486
248
10
Arbitrary Units
0
25
50
75
100
Volume (%)



From the drop-down list, select the curve fitting type that most accurately reflects your data points (as a rule of thumb, this is usually the curve type that has the highest R2 value, the closer to 1 the better).

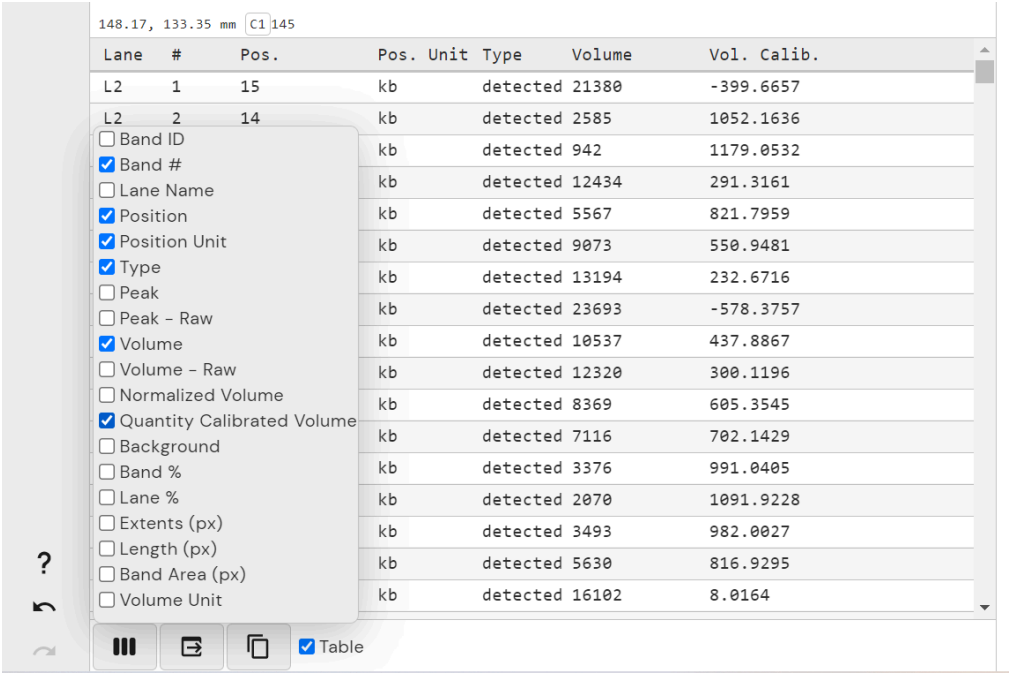
Both the equation used to calculate the curve and the R2 value can be found below the drop-down once curve type has been selected:



> *Whilst at least 3 bands with known values are required to create a calibration curve, Phoretix 1D supports a virtually unlimited number of known band values to create your calibration curve. The greater the number of standards that form the calibration curve the greater the accuracy of that curve and therefore the greater the accuracy of the interpreted (unknown) band values derived from it.*

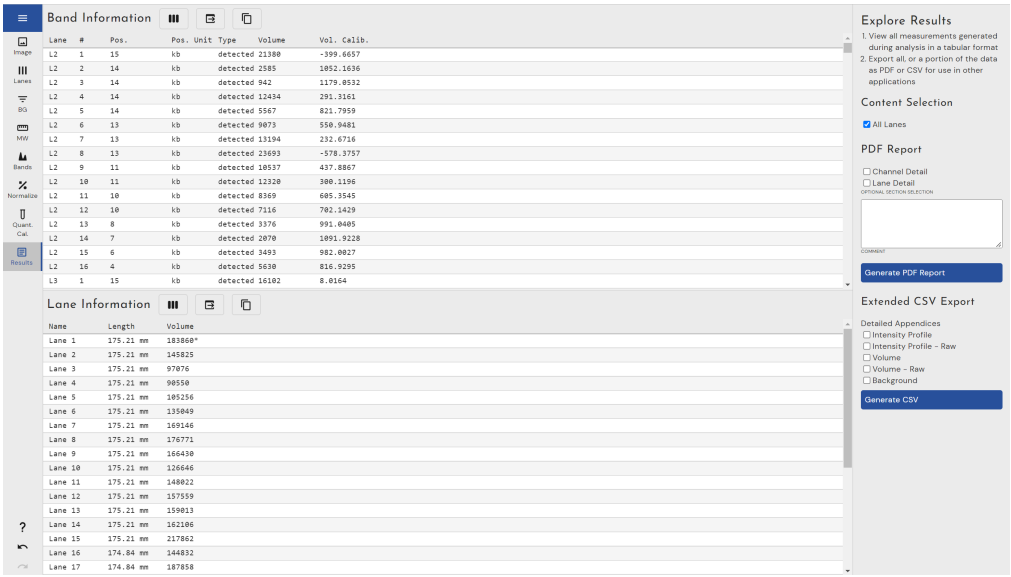
Displaying calibrated band values

To show your quantity calibrated band values, left-click the results table menu and tick the "quantity calibrated volume" check box:



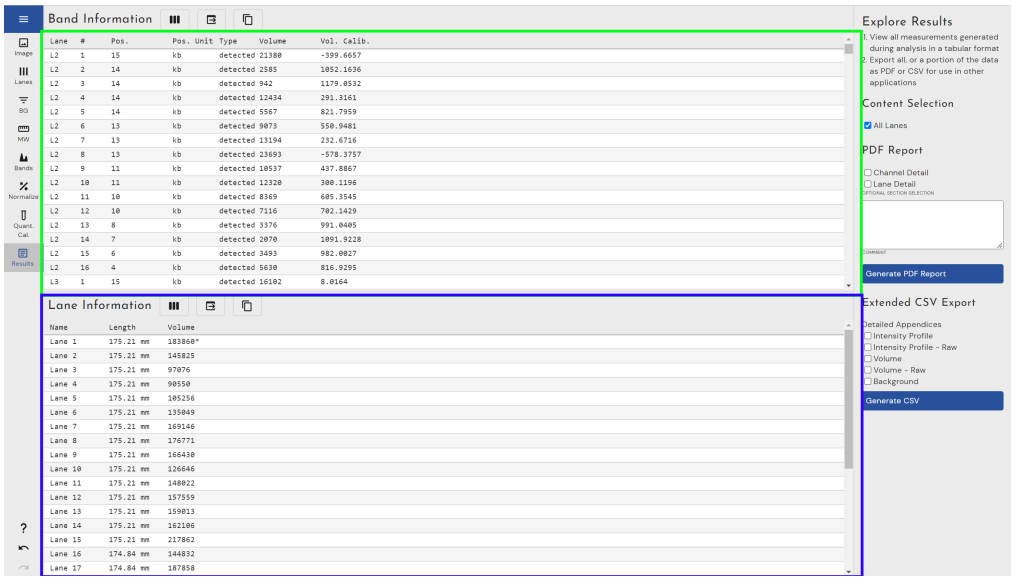
Calibrated band values are then displayed in the "Vol. Calib." column in the results table.

Results Mode



Results mode is the final stage of your analysis, where you are able to gather all of your results together and export them in the form of a report.

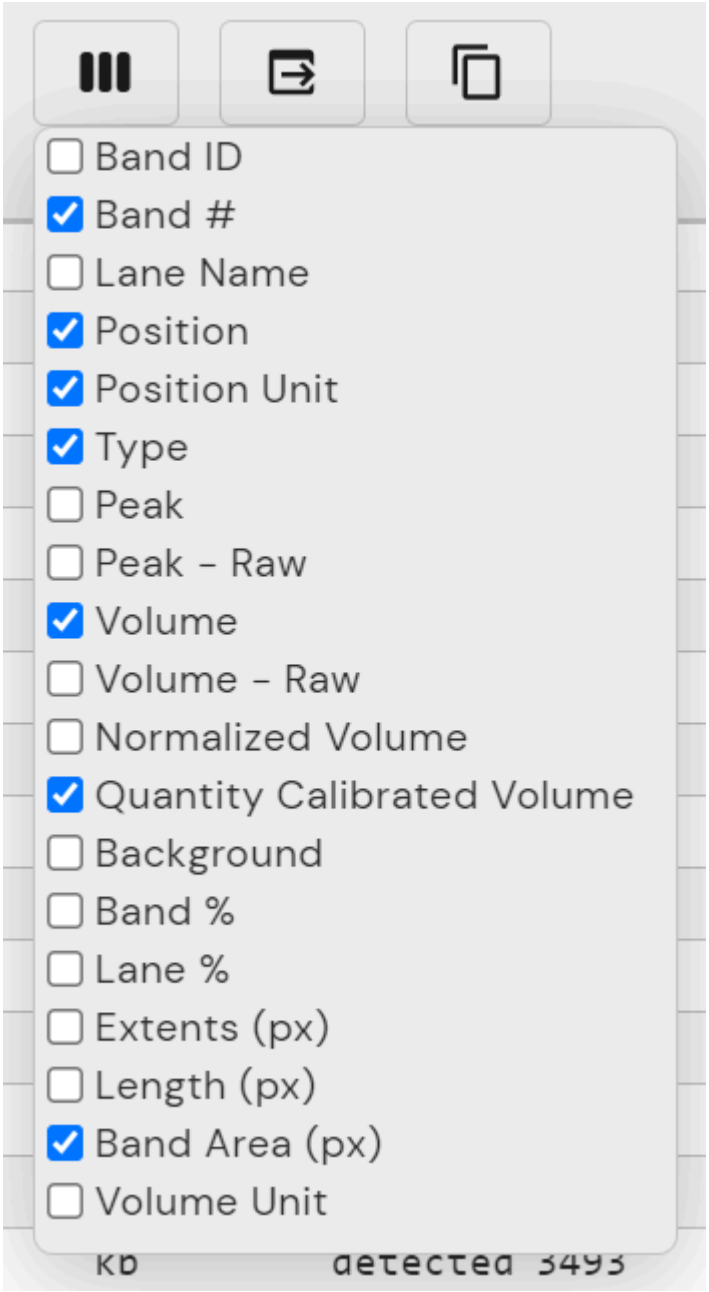
In this mode, your results are seperated into bands (top table, green) and lanes (bottom table, blue):



Each section can be controlled independently using the buttons situated above:

The first is "choose columns":

Which, just like in the other modes of Phoretix 1D, allows the user to choose what columns of results are displayed:



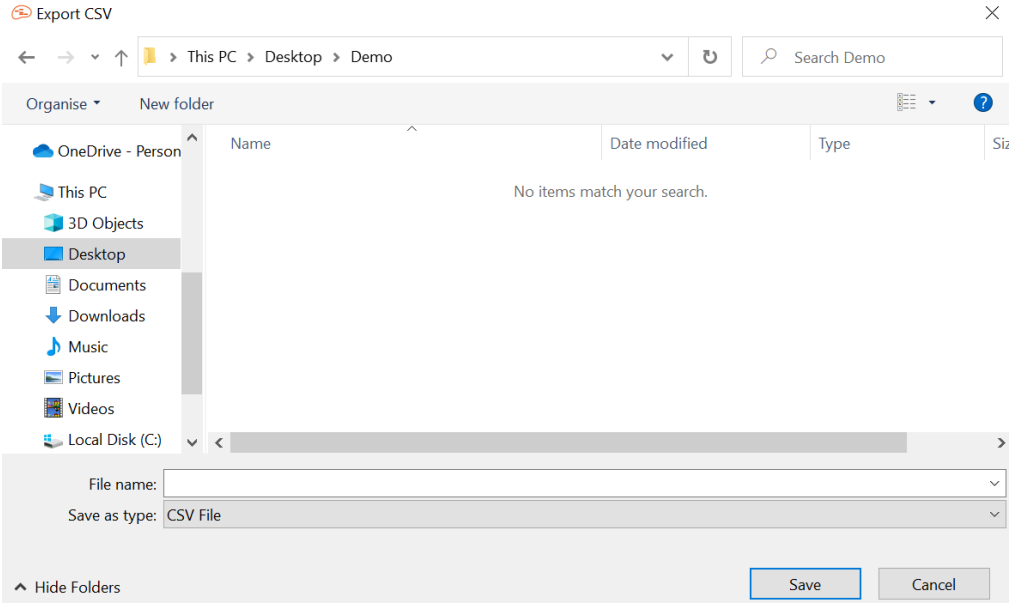
For reference, here is the definition of what each column refers to in the results tables:

- Band ID – Global band ID across the gel. Each band will have a different ID
- Band # – Band number within the given lane (numbered from top of lane down)
- Lane – Lane number the band is present in (numbered left to right)
- Channel – Channel number the band is present in (only available when using a multichannel image AND All Channels checkbox is checked in the right hand taskbar)
- Position – Distance from top of the lane (unit depends on position unit)
- Position Unit – Unit for position (default mm)

- Type – Whether band has been automatically detected, manually added or edited post detection
- Peak – Calculated peak intensity (background removed)
- Raw Peak – Peak intensity including background value
- Volume – Summed volume of pixel intensities in the band box (background removed)
- Raw Volume – Summed volume of pixel intensities in the band box including background value
- Normalized Volume - Normalized volume (if normalization applied)
- Quantity Calibrated Volume - Quantity Calibrated Volume (if Quantity Calibration applied)
- Background - pixel intensity of background for band
- Band % - A measure of the band's Volume divided by the total volume of all the bands in the lane
- Lane % - A measure of the Bands volume divided by the volume of the whole Lane
- Extents (px) – Band start and end points (measured in pixels from the top of the lane)
- Length (px) - Band length (measured in pixels between edge boundaries)
- Band area (px) - Band area (measured in pixels between edge boundaries)
- Volume unit - If using a calibrated image unit used for calibration will be shown, if using an uncalibrated image the units are just intensity

The second is "export CSV" which when left-clicked allows all of the results displayed in the table to be exported in a Microsoft Excel readable .CSV (comma seperated values) file. This is especially useful if you wish to export your results into another program as .CSV is a very widely supported data file type.

Left-click "export CSV" to open a file dialog box that asks you where you wish to save your exported file (the default location is the desktop).



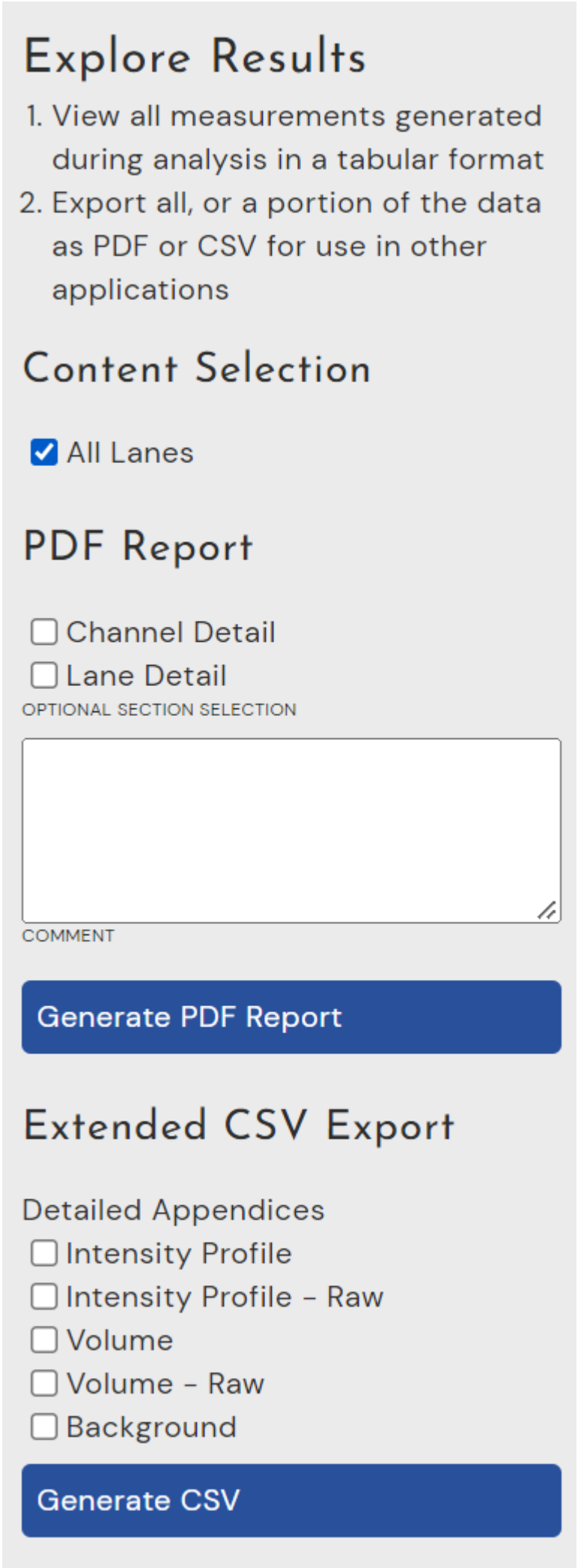
Give your exported file a name and click save.

The final button is "copy to clipboard":

Which copies the current table to your clipboard as an unformatted series of raw values which can then be pasted into a huge number of different applications (Microsoft Excel, statistics software packages etc.).

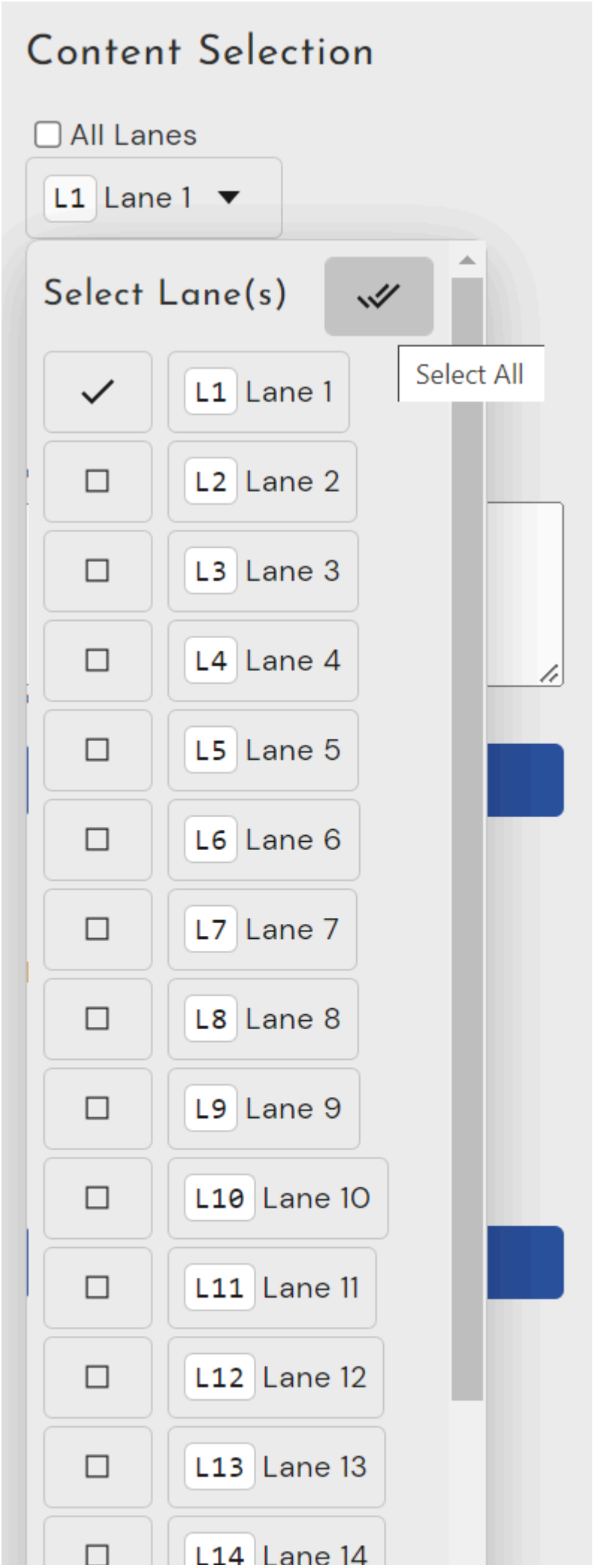
Exporting reports

On the right-hand side taskbar, you can find the options related to report generation:


Explore Results
1. View all measurements generated during analysis in a tabular format
2. Export all, or a portion of the data as PDF or CSV for use in other applications
Content Selection
All Lanes
PDF Report
Channel Detail
Lane Detail
OPTIONAL SECTION SELECTION
COMMENT
Generate PDF Report
Extended CSV Export
Detailed Appendices
Intensity Profile
Intensity Profile – Raw
Volume
Volume – Raw
Background
Generate CSV

Content selection

By default, reports are generated containing the results from all lanes, however by unticking the "All Lanes" checkbox the user can select exactly which lanes they want to include in the report:


Content Selection
All Lanes
L1 Lane 1
Select Lane(s)
Select All
L1 Lane 1
L2 Lane 2
L3 Lane 3
L4 Lane 4
L5 Lane 5
L6 Lane 6
L7 Lane 7
L8 Lane 8
L9 Lane 9
L10 Lane 10
L11 Lane 11
L12 Lane 12
L13 Lane 13
L14 Lane 14

By left-clicking the "lanes" drop-down box the user can select which lanes they want to include within the .pdf report by clicking the checkbox next to the name of the lane. There is also the option from this drop-down to select all lanes by clicking the double tick box in the right hand corner.

PDF Report Options

By default these options are turned off and results in the generation of the most basic type of PDF report when the "generate PDF report" button is clicked.

Included in this report is:

- Greyscale copy of the analysed image
- Date of analysis
- Size of image (in pixels)
- Imager model (if available)
- Date ofaquisition (if available)
- Exposure time used for capture (if available)
- Image displaying analysed lanes
- Lane information including lane name, length and total lane volume
- Image displaying identified bands
- MW ladder name (if used)
- MW ladder steps and pixel position in lane
- Background removal method and settings
- Band detection settings
- Normalisation settings

Channel Detail

Ticking this box includes channel detail to the PDF report, if present in the image metadata

Lane Detail

Ticking this box includes all of the above and adds:

- Lane profiles for every lane
- Band locations within lanes and selected measurements in measurements table

Extended CSV Report

Extended CSV report allows you to export your data as a CSV file with some additional information beyond that exported if you only clicked the "export CSV" button above the band or lane table.

> *Please note: to export a CSV with these values, they need to be turned on in the results tables first*