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Two^{MP} system
Step by step user guide

Refeyn Ltd.

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Two^{MP} system – step to step user guide

1. Turn **ON** the switch that is located at the back of the electronics unit **at least one hour before** starting your experiments.

2. Turn **ON** the Isolation switch which is located at the front of your active vibration isolation table. For that press the buttons "Power" and "Isolation".

3. Open the Acquire^{MP} (AMP) software.

4. Create a new project (you only need to do this at the start of your experiments).

5. Prepare your glass slide using the alignment tool (provided with your Two^{MP} starter kit).
    - Take the alignment tool, place a gasket in the middle, and then place the precleaned glass slide on top. Assure that the gasket is fixed onto the slide by applying light pressure with the back of a pipette tip/tweezer. (https://www.youtube.com/watch?v=J-QE_q97g3M)

6. Open the lid on the Two^{MP} unit. Ensure that the objective and sample carrier are clean (no dried-out oil left on it by the last user). If in doubt, please clean the objective using step 20.

7. Now place a drop of immersion oil (provided in your Two^{MP} starter kit) in the middle of the objective lens. The drop size is sufficient to cover the center of objective but must not flow to the edges of your objective.

8. Now place the glass slide that you prepared in step 5, with gasket facing the top, and place two magnets diagonally at each end of the glass slide.

9. Before you start running your samples, please ensure that your buffer, samples and calibration standards are ready and at room temperature.

10. Select a well (on the gasket) you want to start your experiment on. Use lateral control at speed 50x to move the stage so that the red laser beam is focused on the middle of the selected well. Once this is done, you may now set the well position on the software. This will allow for automatic stage positioning to another well when you run your next sample.

11. Take 10 µl of very clean buffer in a pipette and add it to the selected well in step 10.

12. Close the lid. On the "Focus control" window, press "droplet dilution" button. A window will pop up confirming that the software is now finding focus. Once the window disappears and you see the lock sign turns orange, it means that it has found focus and you can now add your sample to the well.

13. Add 5-10 µl of your diluted sample (100pM- 100nM) and add to it the same well you found focus on. Mix 3-4 times by pipetting up and down.

14. Close the lid. Press "record" button.

15. The software will now record data for 1min. Once the recording is finished, it will ask you to save the video into the project that you created in step 4. The data will be saved as .mp file.

16. Unlock the focus control and select next well you want to use for the new measurements.

17. To run your next sample, repeat steps 9-15.

18. Once you have run 6 measurements (6 wells in the gasket). Prepare and use a new slide with gasket (step 5). Please DO NOT reuse the glass slide.

19. If you are continuing your experiments and just need to change to a new slide and gasket, it is acceptable to do a quick clean of the objective, just wipe the objective with a "Microscope lens cleaning tissue". And then add immersion oil and start working from step 7. However, once you have finished, please ensure to give the objective a thorough clean as mentioned in step 20 below.

20. Now clean the objective using the lens cleaning tissue provided with your starter kit.
    Wipe the excess oil with the tissue. Then take another tissue, fold it well, add some drops of 100% IPA on one end and start cleaning the center moving outwards to the edges. Repeat this 3-4 times until the objective is clean. Ensure that the stage plate is also clean and oil free.
    (https://www.youtube.com/watch?v=HjXowJDQZvE)

Additional Notes

Tip window

AMP software contains a Tip window for this and more information.
The tip window can be prompt simply by clicking the "help" menu and selecting the tips button.

If not using cell culture grade buffers

If your buffers are not cell culture grade, it is likely that your buffers might contain impurities that will interfere with the data (you may note some events in the ratiometric image which you would not see otherwise). If this is the case, please ensure that your buffer is clean by filtering the buffer using a 0.2µm filter.

Seeing huge white spots

If you see huge white spots on the native or ratiometric image, it is likely that your area selected on the slide is dirty, use lateral control and reduce speed to 1x and hit left/right/top/button signs to find a clean area. Find focus again.

Seeing interference patterns

If you see interference patterns in the image (diagonal lines), it indicates that the system is either: a) focused close to the edges of your selected well or b) you have introduced a small air bubble in the sample.

a) Use lateral control, speed 1x and the direction signs to move towards the center. And find focus again. Always unlock the focus before you decide to move the stage using lateral control (otherwise it may impact your stage performance over time).
b) The interference pattern may also be caused due to a bubble in the buffer. If this is the case, pipette up and down to try to remove the air bubble.

Break in the ring

If you see breaks in the ring opposite to each other, there is an air bubble in the oil. To get rid of the bubble, remove one magnet, and gently lift the slide from one side and move it up- down couple of times. And place the magnet back. Close the lid. Find focus again.

If you see a single and multiple breaks at random spots in the ring, report it to support@refeyn.com immediately.

Calculator tool

Use the software calculator (under tools or directly on the top pane in the new software) to check your dilutions.

Field of view

If your proteins/ biomolecules are at a higher mass range, for example >500 kDa, you may choose to work on a larger field of view. Go to acquisition settings, select large, ok.

If you are doing this, please run your mass calibration in large FOV as well.

DMP Software

Creating mass calibration and applying it to your samples

1. Open the Discover^{MP} (DMP) software.

2. Click on + sign to load all your files you saved in AMP software. (For mass calibration, use the measurements run with calibrants (highly purified biomolecules of known mass).

3. Once it loads, it will automatically start processing your movie and generate a graph, it may take a few seconds.

4. You can start working with the first file once it is processed (while the processing for other ones are running in the background).

   The top left panel has all the files you have obtained from your measurements, button left panel is where your mass calibrations will be saved, the middle panel represents the movie/each frame in the movie the software is using to process your data, and the right panel is the histogram of all events that are captured from the movie.
   The histogram that you see will appear with ratiometric contrast. In order to convert it to mass, we will need to have a mass calibration and apply to your measurements.

5. The software will automatically fit peaks. If all peaks look relevant to you, move to the next step. Otherwise, click clear at the top, and all the peaks will disappear. You can then double click on the peak that seems relevant to your sample.

6. You could also set contrast limits as you wish.

7. Once the peaks are fit, press "mass calibration" button.

8. A window will pop up with contrast values for all peaks already added. Set mass for each value and save. If there is only one peak/point, add another point and set mass and contrast as 0, which will then allow you to save the calibration.

9. This will then create a calibration file at the button pane, which you can rename, and then export using the arrow sign at the top of this pane. This will be saved as .mc file.

10. Repeat steps 5-8 with your second measurement.

11. To add saved calibrations points on the current calibration, click append from file and load the exported calibration file from step 9. This will then show you a straight line with a mass error. Mass error shouldn't be more than 5%. If it is, check if the MW molecular weights are well attributed to the contrast, if the gaussian fitted in the peaks captures the particle distribution measured. If there is no obvious mistake on the previous you might need to repeat your experiments.

12. In total you may want to get three points for mass calibration, it could be from one protein complex giving three points, or two independent measurements, one giving two points and the other one giving one point or so on.

13. Rename and export the calibration file, same as step 9.

14. Right click on the calibration file and click apply to all. Or select the measurement you want to apply it to and double click on it. You can now see your graphs in mass.

Sample analysis

15. To analyse your own samples, follow steps 2-6, and apply mass calibration to it as per step 14.

16. You can also generate figures by simply clicking on Add figure, drag your files to the figures pane. You could overlap multiple files or work on them separate simply by adding figure.

17. There are several options provided on the software to make your graph look publication perfect. Make changes as you please. And then export figure in .png, .svg, .pdf, or .eps file types.

Saving your analysis

You can save each file separately or save it as a workspace (all files saved at the same time). These processed files will be saved in .dmp file type. This step is helpful as you can then open already processed data and not repeat the above steps again.