

Bradford method

Mitochondrial protein concentration in the final solution obtained at the end of the isolation protocol can be determined using the Bradford method. This step is crucial to normalize the results that you will obtain during further experiments. The following protocol is an example and can be modified or improved depending on your experimental conditions. Keep in mind that your final solution of mitochondria needs to be kept at 4°C!

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1. Prepare a BSA standard solution

Dilute, if necessary, a standard solution of BSA in pure water to obtain a solution containing $200\mu\text{g/ml}$ of BSA. This diluted solution of BSA will be used to determine a calibration curve. Prepare 6 tubes using the following protocol:

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Tubes (n°)	BSA to add (μl)	Water to add (μl)	Final quantity of BSA (μg)
1	0	1000	0
2	10	990	2
3	25	975	5
4	50	950	10
5	75	925	15
6	125	875	25

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2. Make a dilution of your mitochondrial solution

If your mitochondria have been isolated following the «isolation of mitochondria» section, your final solution is highly concentrated. You will therefore have to make a diluted solution of your final mitochondrial solution to be able to determine the mitochondrial protein concentration using the Bradford method. The dilution factor that you have to apply depends on the quality of your preparation and the tissue you are studying. The following table gives some examples of dilution factor ranges you can use for several tissues (but you

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have to adapt this dilution factor to your own experimental conditions to obtain a diluted solution at a concentration of approximately 1 - 1,5 mg / ml):

Tissue	Dilution factor
Skeletal muscle	25 - 30
Heart	30 - 40
Liver	30 - 35

To make this diluted solution, take 10 μ l of your final mitochondrial solution (using a Hamilton seringe to be accurate), add these 10 μ l to a new tube, and fill in this tube with the required amount of pure water

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corresponding to the dilution factor that you want to apply.

When this dilution is performed, mix the resulting solution for 2 minutes. In pure water, mitochondria will swell, therefore leading to the disruption of inner mitochondrial membrane and consequently to the release of matrix proteins in the water. To ensure a correct homogenization of your sample, you can also sonicate your diluted mitochondrial preparation for 5 minutes. You won't have to use again the final solution of mitochondria. So don't forget to keep it on ice until their utilization for functional assays!

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Your diluted solution is now ready, and you therefore can prepare the Bradford assay!

3. Prepare the assay

In order to accurately determine the mitochondrial protein concentration, three assays will be performed to minimize experimental errors. The diluted solution you prepared is still too highly concentrated, but has a suitable concentration to prepare the assay using the protocol presented in the following table:

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Tubes (n°)	Diluted Solution of mitochondria (μ l)	Water to add (μ l)
7	0	1000
8	10	990
9	15	985
10	20	980

You have therefore performed a second dilution of the final solution of extracted mitochondria.

All your samples are now ready for the bradford assay.

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4. Add the commassie reagent

At this step, you have prepared 10 tubes to which you will have to add a coomassie protein assay reagent. It is important to note that your sample needs to be incubated with this reagent for at least 10 minutes to ensure a stable and accurate signal.

Therefore, with the help of a timer (and why not using the timer of this app!) start to add 1 ml of the reagent to the tube n°1, mix carefully. 30s after, add 1ml of the reagent to the tube n°2 and mix carefully, etc... til tube n°10.

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5. Spectrophotometrical assays

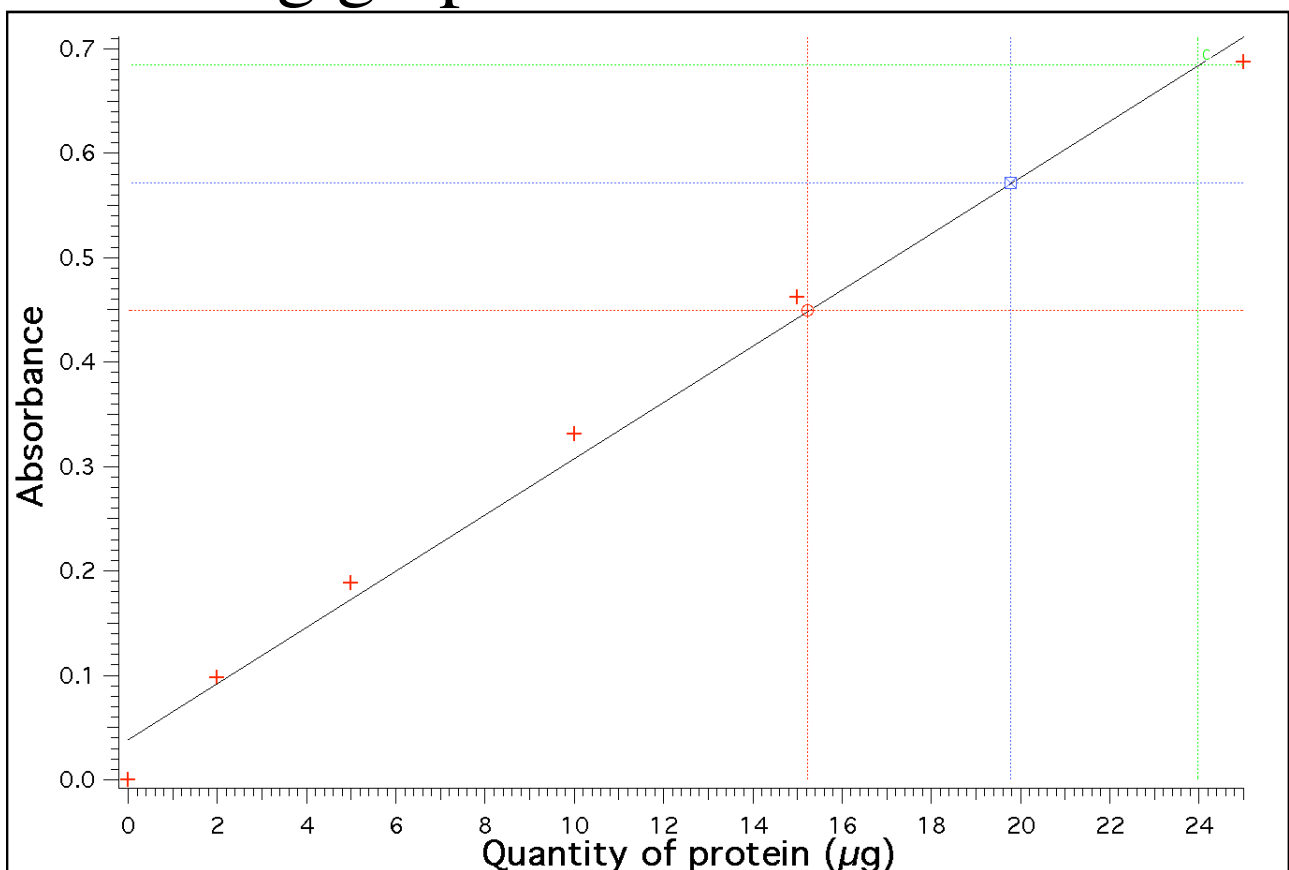
When your timer indicates 10 min, it's time for you to start the spectrophotometrical determination of protein concentration. the wavelength of the spectrophotometer needs to be set to 595 nm. Place the content of tube n°1 in a cuvette and place it into the spectrometer chamber to scale the absorbance to 0. Thirteen seconds after, put the content of tube n°2 in a cuvette and place it into the spectrometer chamber, and collect the corresponding absorbance, etc... until tube n°10.

Tube n°1 to 6 will be used to determine the calibration curve. Tube n°7 will be used to ensure that without sample, the

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signal obtained is null or negligible. Tube n°8 to 10 will be used to determine the protein concentration in your sample.

Using this protocol, you will obtain the following graph:



Graph 1: Example of Bradford results. Red cross correspond to the different

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points of the calibration curve and the circles correspond to the three points of the sample assays (tubes n°8 to 10).

1. How to determine the concentration of your final solution of mitochondria?

As you can see on the previous graph, this protocol allows the determination of the quantity of protein you have in the tubes n°8 to n°10. Consequently, the bradford assay gives you the quantity of protein contained in 10, 15 and 20 μ l of your diluted solution. So, you can easily determine the concentration of the diluted solution of mitochondria prepared during step 2.

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Average the three different results that you will obtain to minimize experimental errors. Consequently, the concentration of the final mitochondrial solution can be determined by multiplying the concentration of the diluted solution by the dilution factor you applied during step 2.

1. Example

Here is an example corresponding to the graph shown in step 5.

As you can see on this graph, the quantity of protein contained in the tubes n°8 to 10 were $14.6 \mu\text{g}/10\mu\text{l}$, $18.9 \mu\text{g}/15\mu\text{l}$ and $20.4 \mu\text{g}/20\mu\text{l}$, respectively.

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Express these results as $\mu\text{g}/10\mu\text{l}$ as follow:

Tube n°8 : $14,6 \mu\text{g}/10\mu\text{l}$

Tube n°9 : $18.9/1.5 = 12.6 \mu\text{g}/10\mu\text{l}$

Tube n°10 : $20.4/2 = 10.20 \mu\text{g}/10\mu\text{l}$

Average these three results to obtain the concentration of the diluted solution of mitochondria ([Dmito]):

$$[\text{Dmito}] = (14.6 + 12.6 + 10.2)/3 = 12.47 \mu\text{g}/10\mu\text{l}$$

Express the [Dmito] as $\mu\text{g}/\text{ml}$ as follow:

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$$[\text{Dmito}] = 12.47 * 100 = 1247 \mu\text{g/ml}$$

To determine the concentration of the final solution of mitochondria ([Final Mito]), you have then just to multiply this result by the dilution factor you applied to this solution. In this example, dilution factor used was 35:

$$[\text{Final Mito}] = (1247 * 35)/1000 = 43.6 \text{ mg/ml}$$

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The determination of protein concentration of your final mitochondrial solution is now finished! You can now use your mitochondria for functional assays!

2. Reference

1. Bradford, M.M., *A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding*. Anal Biochem, 1976. **72**: p. 248-54.