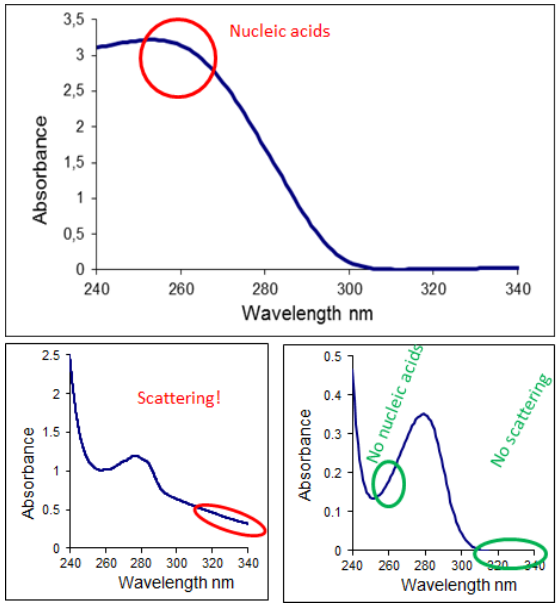
(iii) Extended quality control parameters

Depending on the intended use and in addition to the methods listed above, the following methods may also be applicable and are highly recommended, although they are not considered to be as essential as those in section (ii)

The first of these tests can be performed simultaneously when determining protein concentration spectrophotometrically (using A_{280nm}) as most instruments will also allow you to collect data over a wide spectrum of wavelengths. This additional data can be very informative.

- **General quality test by UV spectroscopy** between 200nm and 340nm to check nucleic acid content and general protein fitness/quality: **Mandatory if protein binds nucleic acids.**



As general quality test the simplest qualitative test is the measure of UV absorbance between 240nm and 340nm. One of the main advantages is the availability of such equipment in all the biological laboratories. Apart from measuring the concentration by means of the protein extinction coefficient, the full spectrum will inform on the general quality of the preparation. It should be stressed that the exact protein buffer as to be used as a blank in order As general quality control test the simplest qualitative test is the measurement of UV absorbance between 240 nm and 340 nm. One of the main advantages is the availability of such equipment in all the biological laboratories. Apart from measuring the concentration by means of the protein extinction coefficient, a full spectrum will inform on the general quality of the preparation as well. It should be stressed that the exact protein buffer needs to be used as a blank in order to avoid errors in the interpretation. A strong signal at 260 nm is usually a sign of nucleic acid contamination. As a general rule the ratio between the absorbance at 260 nm and 280 nm (Abs 260/Abs 280) should give a value close to 0.6 for a good quality protein preparation. Furthermore, a regularly rising absorbance between 340 nm and 300 nm is usually the sign of scattering due to aggregation (see figure). One simple way to confirm scattering is to determine an aggregation index by calculating 100 x Abs 340/[Abs 280 - Abs 340]. As a rule of thumb, an index lower than 2 would be acceptable for a non-aggregated protein.
More detail about UV spectrometry can be found in:

- *Leach SJ, Scheraga HA: Effect of Light Scattering on Ultraviolet Difference Spectra. J Am Chem Soc* ***1960, 82:4790–4792.***

- *Validity of nucleic acid purities monitored by 260nm/280nm absorbance ratios. Glasel JA. Biotechniques.* ***1995; 18:62-3.***
- *Dual effects of Tween 80 on protein stability. Wang W1, Wang YJ, Wang DQ. International Journal of Pharmaceutics* ***2008; 347:31–38***
- *Monodispersity of recombinant Cre recombinase correlates with its effectiveness in vivo. Capasso P, Aliprandi M, Ossolengo G, Edenhofer F, de Marco A BMC Biotechnol* ***2009; 9:80***

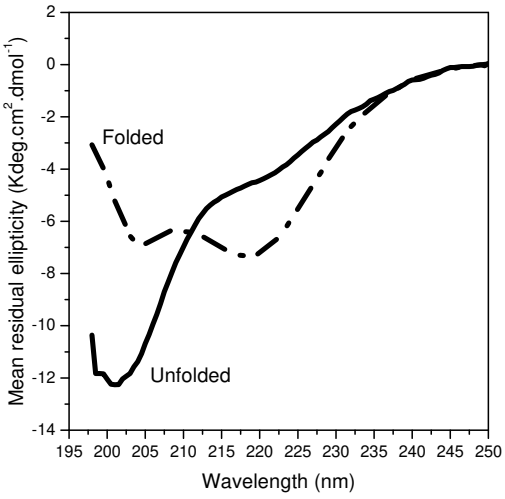
- **Homogeneity:** The following techniques are complementary to the ones previously described: analytical Ion Exchange Chromatography (IEX), analytical Hydrophobic Interaction Chromatography (HIC) or Isoelectric Focusing (IEF). The objective is to get extra information on the homogeneity of the sample. If you need a full description of the techniques please see:

- Separation techniques: Chromatography Ozlem Coskun North Clin Istanb. **2016; 3: 156–160.**
- Isoelectric Point Separations of Peptides and Proteins. Pergande MR, Cologna SM. Proteomes. **2017; 5: E4**

- **Conformational stability/folding state:** Circular Dichroism (CD), Differential ScanningCalorimetry (DSC), NMR, Fourier Transform InfraRed (FTIR). The objective of the measurement is to verify that the same folding signature can be seen. One of the classical technique to perform this type of measurement is to use circular dichroism.

Example: Folding state by circular dichroism

Circular dichroism (CD) looks at the difference of absorption of left and right handed light. In the figure, typical curves of both an unfolded protein (plain line) and of a folded protein (dotted line) are presented.

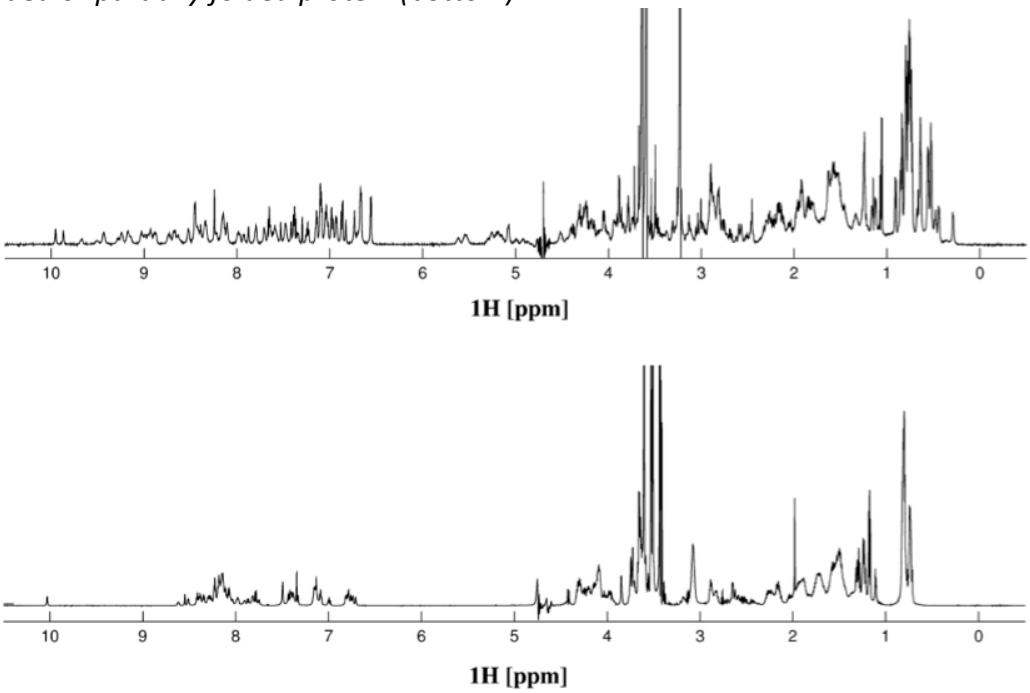


More detail about CD can be found in:

- *How to study proteins by circular dichroism. Kelly SM1, Jess TJ, Price NC. Biochim Biophys Acta.* ***2005; 1751:119-39.***
- *Woody WR. Methods in Enzymology* ***1995; 246****:*
- *http://www.proteinchemist.com/cd/cdspec.html*
- *Circular Dichroism and the conformational analysis of biomolecules. Fasman GD, Plenum Press,* ***1997*** *New York and London*
- *Circular dichroism and its application to the study of biomolecules. Martin SR, Schilstra MJ. Methods Cell Biol.* ***2008; 84:263-93.***

Example: Folding state by NMR spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy is a spectroscopic method to study magnetically active nuclei in an external magnetic field. Measuring a one dimensional (1D) ^{1}H NMR spectrum of a protein requires only little material, is pretty fast and the spectrum clearly indicates if a protein is folded or not. In the 1D ^{1}H spectrum of a well-folded protein (top) the peaks are narrow and sharp and distributed over a large range of chemical shifts (good signal dispersion); signals can be especially found at ppm values < 0.5 (corresponding to high field-shifted methyl group protons) or > 8.5 (corresponding to down field-shifted amide protons) are observable. In contrast, the peaks are broader and not as widely dispersed in the spectrum of an unfolded or partially folded protein (bottom).



More details about NMR spectroscopy can be found in:

- *Applied NMR Spectroscopy for Chemists and Life Scientists. Zerbe O, Jurt S. Wiley-VCH Verlag GmbH & Co. KGaA,* ***2014*** *Weinheim, Germany*

- **Protein competent fraction**, i.e. the relative amount of functionally active protein, measured as specific activity, by active site titration or other suitable methods. The objective of this test is to be able to determine the amount of active molecule in the protein preparation. When the specificity test is based on interaction between molecules this measure can be realized by a surface plasmon resonance (SPR) technique using the "calibration-free concentration analysis" (CFCA) method, which has been implemented in different SPR instruments available commercially. More detailed can be found in:

- *Pol E: The importance of correct protein concentration for kinetics and affinity determination in structure-function analysis. J Vis Exp* ***2010, 37:2–8****.*

- **Endotoxin content**, this analysis is mandatory for protein samples used in combination with cell cultures.

- **Optimization of storage conditions**: To minimize the formation of protein aggregates and to improve solubility, adjustment of several parameters of the sample buffer composition (pH, salinity, the presence of detergents, cryo-protectants or other additives, co-factors or ligands etc.) can be made to increase homogeneity and long term stability. These conditions can be assessed using automated technique such as DLS (described above) or thermal shift assay.

Example: optimization of buffer condition by automated DLS

This DLS conformation that allows processing a large number of samples in plate format, has simplified buffer condition screening. Buffer matrices for multi-parametric screening of pH, salinity, buffer nature, additives and co-factors can be generated by hand or using simple robotics. One approach is to dilute samples in the different buffer, classically ten times with a final concentration of 1 mg/ml for a 10 kDa protein or 0.1 mg/ml for a 100 kDa protein. The homogeneity of the sample and the presence of aggregates can be assessed for each condition, and the optimal buffer composition can be selected, according to solubility parameters and the downstream application.

More detail about this approach can be found in:

- Solubility at the molecular level: development of a critical aggregation concentration (CAC) assay for estimating compound monomer solubility. Wang J, Matayoshi E **Pharm Res 2012, 29:1745–1754.**

Example: optimization of buffer condition by thermal shift assay

Modern Thermofluor and differential scanning fluorimeter that allow processing a large number of samples are methods for screening buffer conditions. Similarly to automated DLS, buffer matrices can be generated.

In a thermal shift assay, protein unfolding is monitored by heating up the samples in a linear temperature gradient in the presence of an environmentally sensitive dye. One of the most commonly used dyes is SYPRO orange, which fluoresces upon interaction with hydrophobic parts of the protein. As the temperature increases, the protein starts to unfold and exposes its hydrophobic core. The dye interacts with these exposed hydrophobic areas and the fluorescence increases. Ideally, the samples will display a sharp increase in fluorescence over a short temperature interval. The inflection point of this sigmoidal curve then represents the protein melting temperature T_m, at which 50% of the protein is unfolded. Most thermal shift assays are performed by using 96-well plates containing various buffer conditions in a real-time PCR machine. Comparing the T_m´s between all these different conditions provides hints to optimize the buffer conditions for protein purification and storage.

More detail about this approach can be found in:

- Optimization of protein purification and characterization using Thermofluor screens. Boivin S, Kozak S, Meijers R: **Protein Expr Purif 2013, 91:192–206.**

- A thermal stability assay can help to estimate the crystallization likelihood of biological samples. Dupeux F, Röwer M, Seroul G, Blot D, Márquez JA. Acta Crystallogr D Biol Crystallogr. **2011; 67:915-9.**
- Leung S-M, Senisterra G, Ritchie KP, Sadis SE, Lepock JR, Hightower LE. Thermal activation of the bovine Hsc70 molecular chaperone at physiological temperatures: physical evidence of a molecular thermometer. Cell Stress & Chaperones. 1996;1(1):78-89.

- **Batch-to-batch consistency: Mandatory if more than one batch is used-many factors can affect the quality of your protein and you should never assume that all preps are of equal quality.** Use some of the methods listed above, e.g. spectroscopic technique such as UV spectroscopy and circular dichroism are rapid and effective methods of quality assessment.