# Stability of biomolecules - methods

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*Autumn 2023*

S1004 Methods for structural characterization of biomolecules

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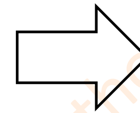
# Stability

Resistance in environment

- Capability to retain **native structure** (stay folded)
- Capability to retain **activity**

no structure



no activity

structure

? activity

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# Stability

## Quick reminder – structure hierarchy

| | Protein | DNA |
|---|---|---|
| Primary | Sequence (aminoacids, N-term - C-term) | Sequence (nucleotides, 5`- 3`end) |
| Secondary | α-helix, β-sheet, turns, loops (rotation along torsion angels Ψ and Φ) | Watson-Crick base pairing (A-T, C-G) |
| Tertiary | 3D organization of secondary motives | A-form, B-form, Z-form |
| Quarternary | oligomerization | nucleosomes |

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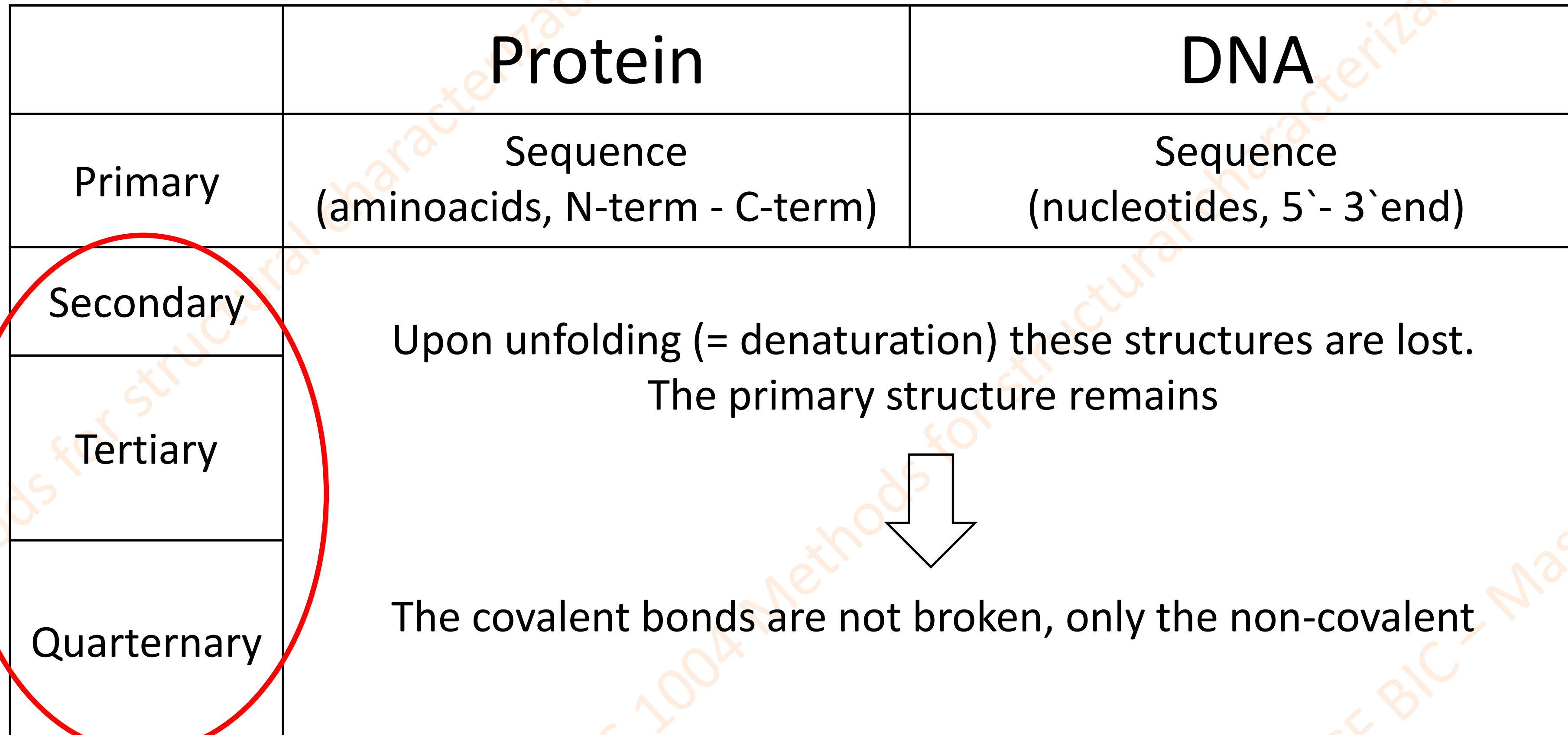
# Stability

Quick reminder – structure hierarchy



| | Protein | DNA |
|---|---|---|
| Primary | Sequence (aminoacids, N-term - C-term) | Sequence (nucleotides, 5`- 3`end) |
| Secondary | Upon unfolding (= denaturation) these structures are lost. The primary structure remains ⇩ The covalent bonds are not broken, only the non-covalent | |
| Tertiary | | |
| Quarternary | | |

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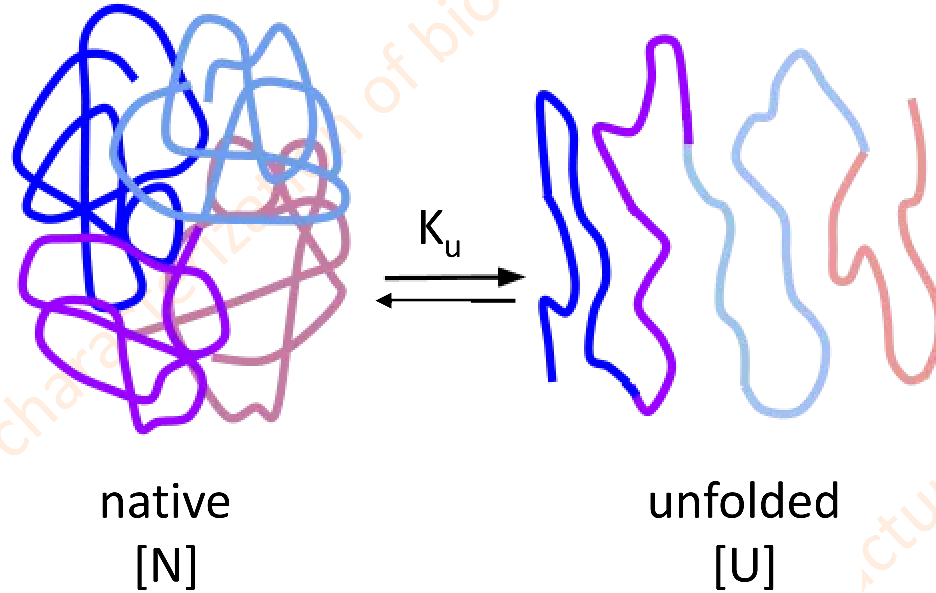
# Thermodynamic consideration




$$K_u = \frac{[U]}{[N]}$$

$K_u << 1$ native state is favourable

$K_u >> 1$ unfolded state is favourable

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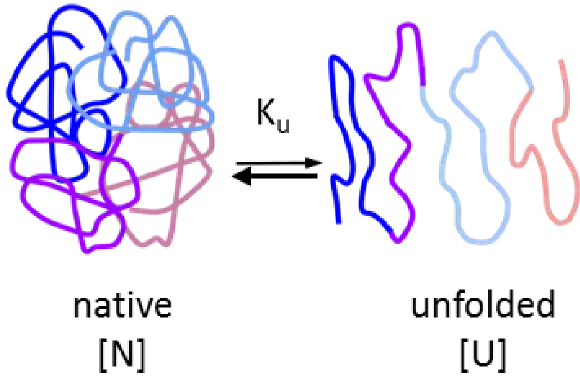
# Thermodynamic consideration



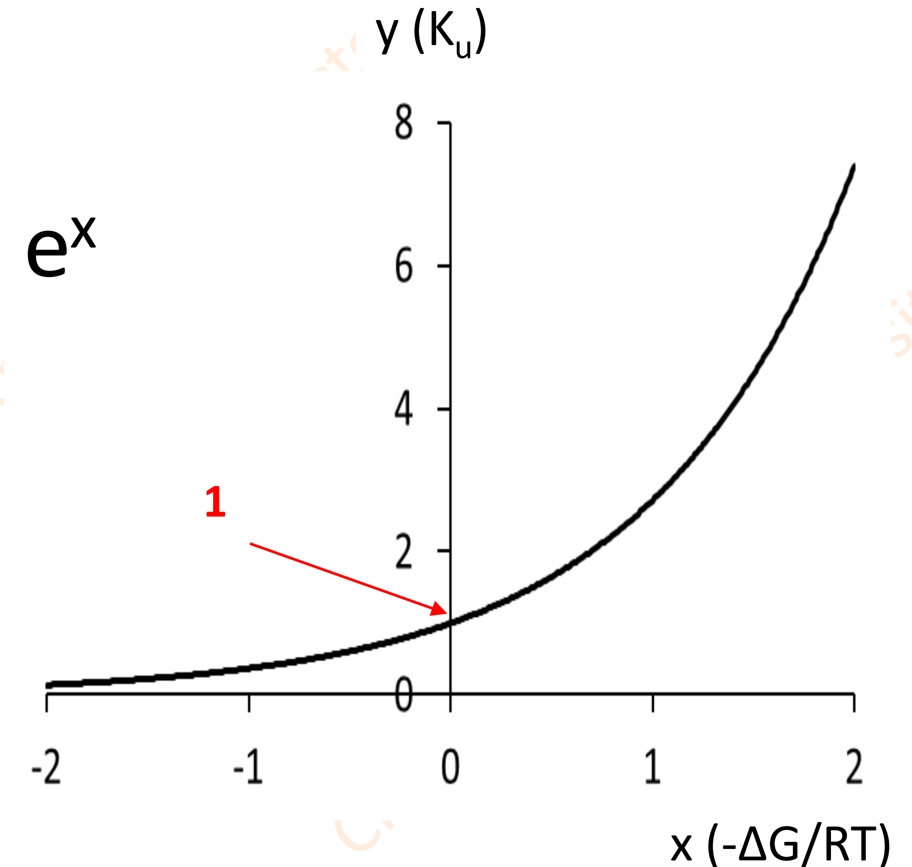

$$K_u = \frac{[U]}{[N]}$$

$K_u << 1$ native state is favourable

$K_u >> 1$ unfolded state is favourable

$$K_u = e^{-\Delta G/RT}$$

$e^x$




Native state is favourable when $K_u < 1$
$-\Delta G/RT$ is negative

$\Delta G$… Gibbs free energy
T… temperature (in Kelvins)
R… gas constant (8.314 $J \cdot K^{-1} \cdot mol^{-1}$)
e… Euler number (2.718….)

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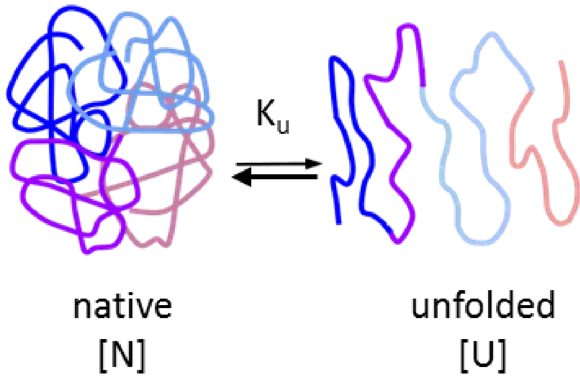
# Thermodynamic consideration




$$K_u = \frac{[U]}{[N]}$$

$K_u << 1$ native state is favourable

$K_u >> 1$ unfolded state is favourable

$$K_u = e^{-\Delta G/RT}$$

Temperature [K] → always positive

Gas constant = 8.314

Gibbs free energy

-ΔG/RT is negative when ΔG > 0

ΔG… Gibbs free energy
T… temperature (in Kelvins)
R… gas constant (8.314 $J \cdot K^{-1} \cdot mol^{-1}$)
e… Euler number (2.718….)

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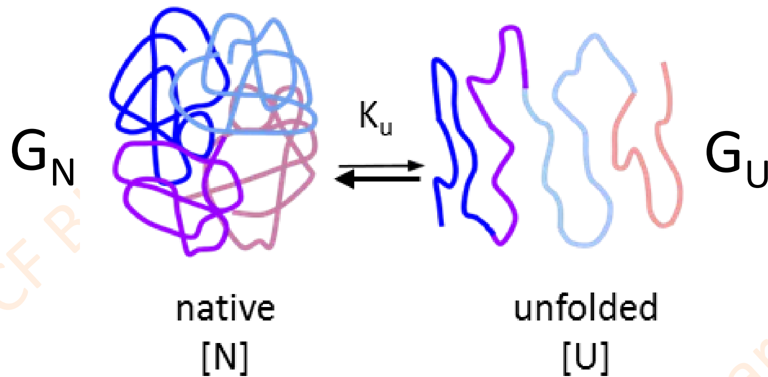
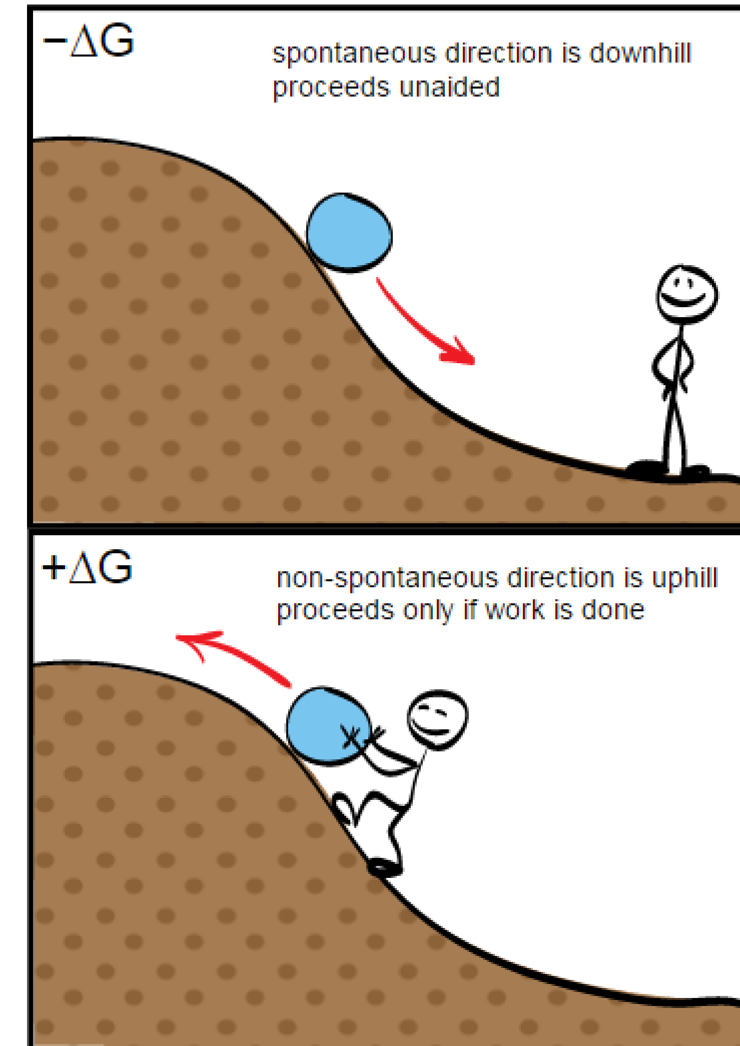
# Thermodynamic consideration




$\Delta G$ = Gibbs free energy

$$\Delta G = G_U - G_N$$

$$\Delta G = \Delta H - T\Delta S$$




The reaction with $-\Delta G$ will hapen spontaneously
The state with lower G is prefered

For the protein to be stable, $\Delta G$ of unfolding needs to be positive (in such a case $G_N < G_U$)

G... Gibbs free energy T... temperature (in Kelvins) H... enthalpy S... entropy

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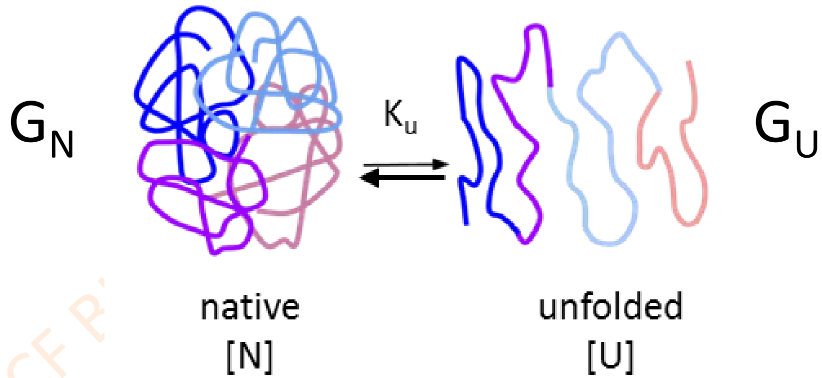
# Thermodynamic consideration




$$\Delta G = \Delta H - T\Delta S$$

$$\Delta G = G_U - G_N$$

## H = Enthalpy

Changes in **heat**

Energy content of the bonds broken and created

hydrogen bonds, van der Waals, salt bridges, S-S

ΔH is negative when bonds are formed

## S = Entropy

Changes in **disorder**

Degree of freedom of molecular movement

Brown`s motion

↑ movement = ↑ S

ΔS is negative when bonds are formed

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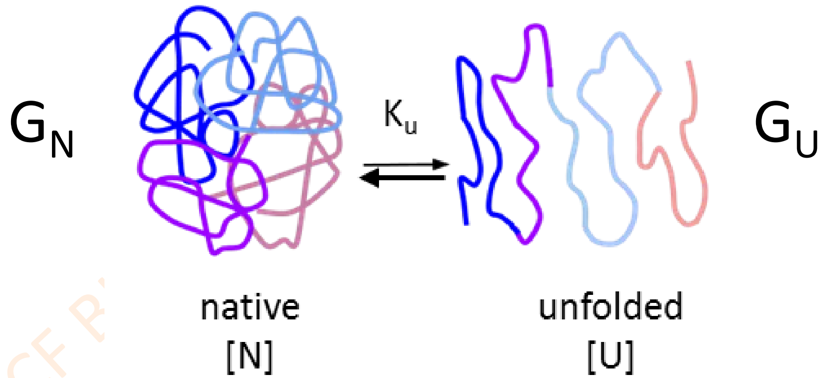
# Thermodynamics of unfolding




**H = Enthalpy**

**S = Entropy**

$$\Delta G = \Delta H - T\Delta S$$

$$\Delta G = G_U - G_N$$

**+ΔS** – unfolded state is more flexible

**+ΔH** – a lot of non-covalent interactions in folded state

**-ΔS** from hydrophobic effect – upon exposure of hydrophobic side chains, the water surrounding the protein forms an ordered cluster („icebergs“)

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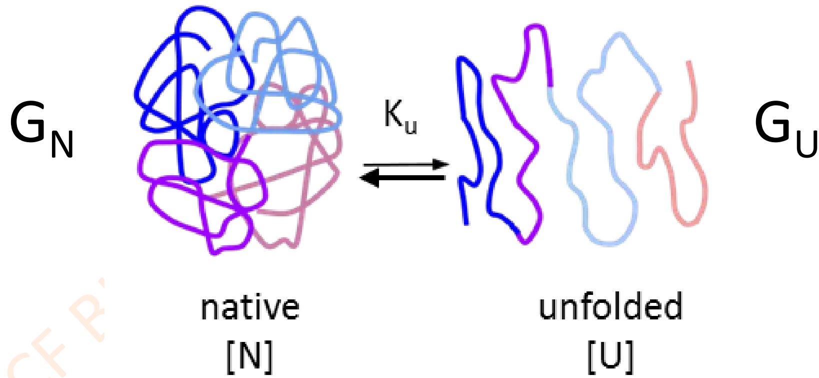
# Thermodynamics of unfolding




$$\Delta G = \Delta H - T\Delta S$$

$$\Delta G = G_U - G_N$$

**H = Enthalpy**

**S = Entropy**

Proteins are just stable

$\Delta G$ of unfolding is typically < 100 kJ $mol^{-1}$
(compared to energy of C-C bond 300 kJ $mol^{-1}$)

Small changes in protein environment can significantly influence the stability

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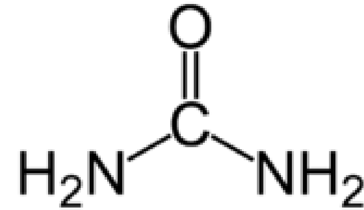
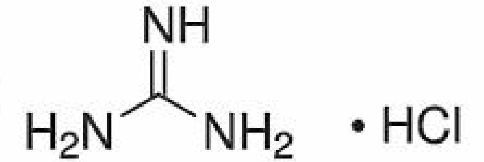
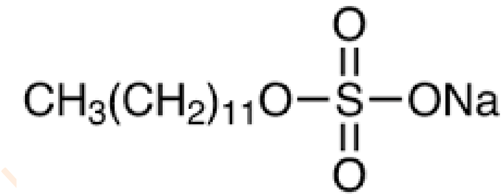
# Denaturing conditions

- Chemicals
  - Urea (around 8M)
  - Guanidium chloride (around 6M)
  - High salts concentration
  - SDS




- Extreme pH
  - Proteins are stable the most near their isoelectric point (pI)

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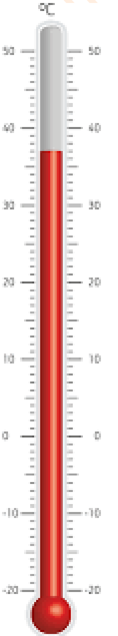
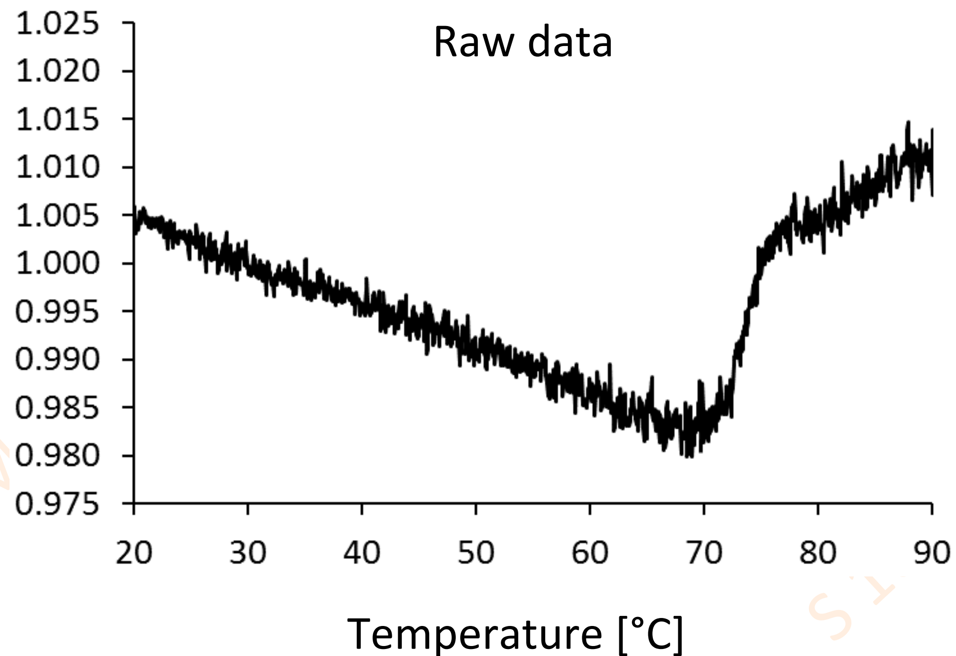
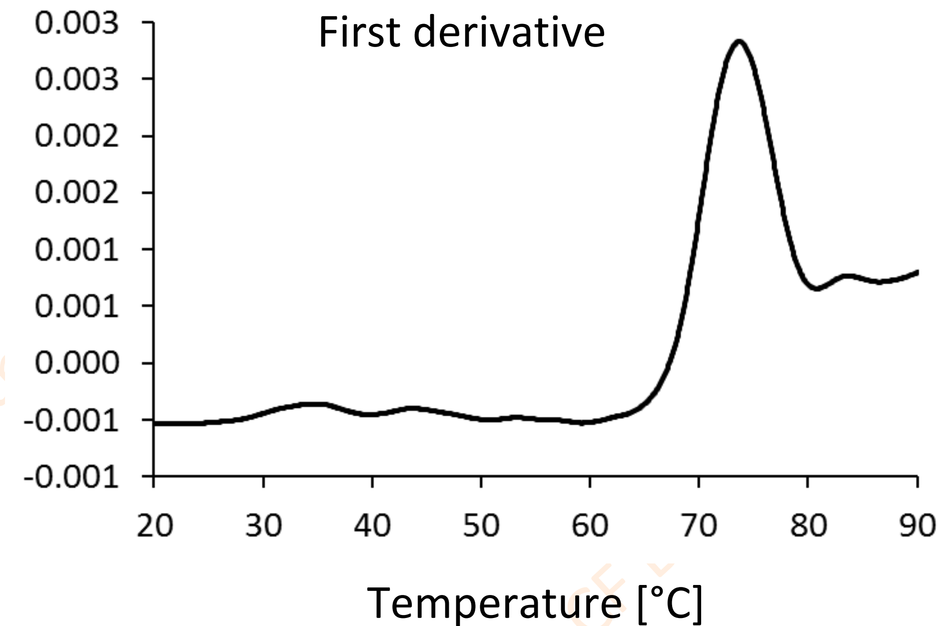
# Temperature as denaturant

$T_m$ = melting temperature

- Temperature at which 50% of the sample is unfolded
- The most reliable indicator of thermal stability



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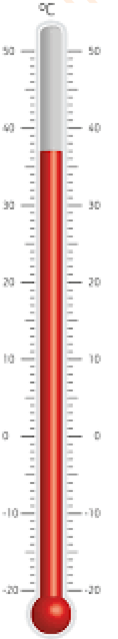
# Temperature as denaturant

$T_m$ = melting temperature

Influenced by:

- Enviroment (buffer, pH, salts):
  in different condition, ΔG of unfolding is different
- Presence of ligand:
  protein-ligand complex is more stable than protein itself
- Heating rate of experiment: slower heating → lower $T_m$
  standard is 1 °C/min



$$\Delta G = \Delta H - T\Delta S$$

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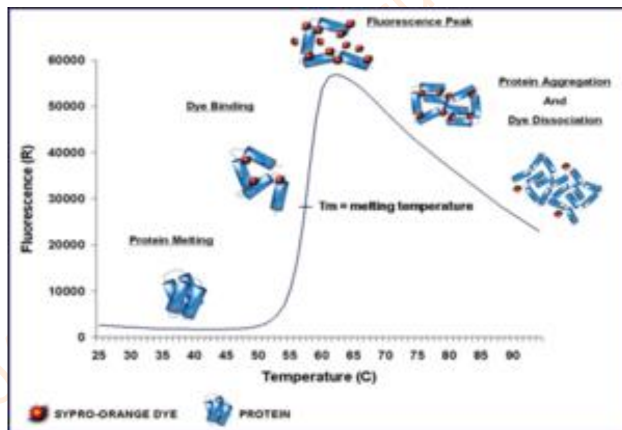
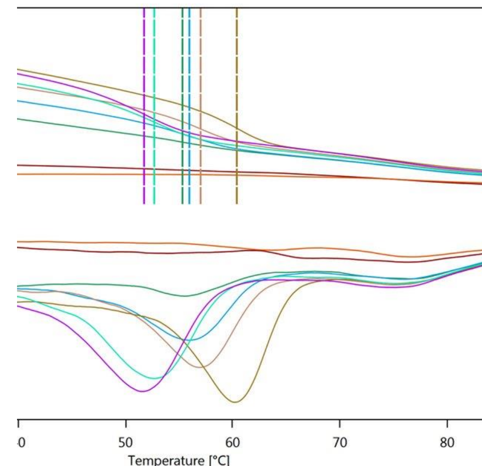
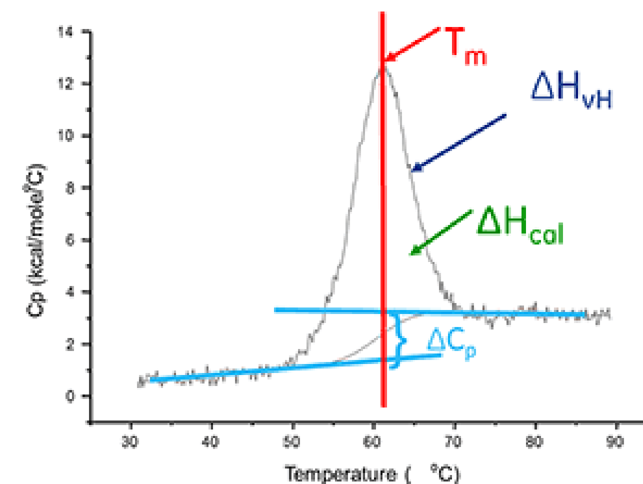
# Methods

- Differential scanning calorimetry (DSC)
- Differential scanning fluorimetry (DSF) – Thermal shift assay (TSA)
- Nano-differential scanning fluorimetry (nanoDSF)
- Circular dichroism (CD)

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# DSC = Diferential Scanning Calorimetry

- Measures the energy absorbed or released by a sample as it is heated or cooled
- Gold standard for $T_m$ determination
- Directly measures the thermodynamic of unfolding

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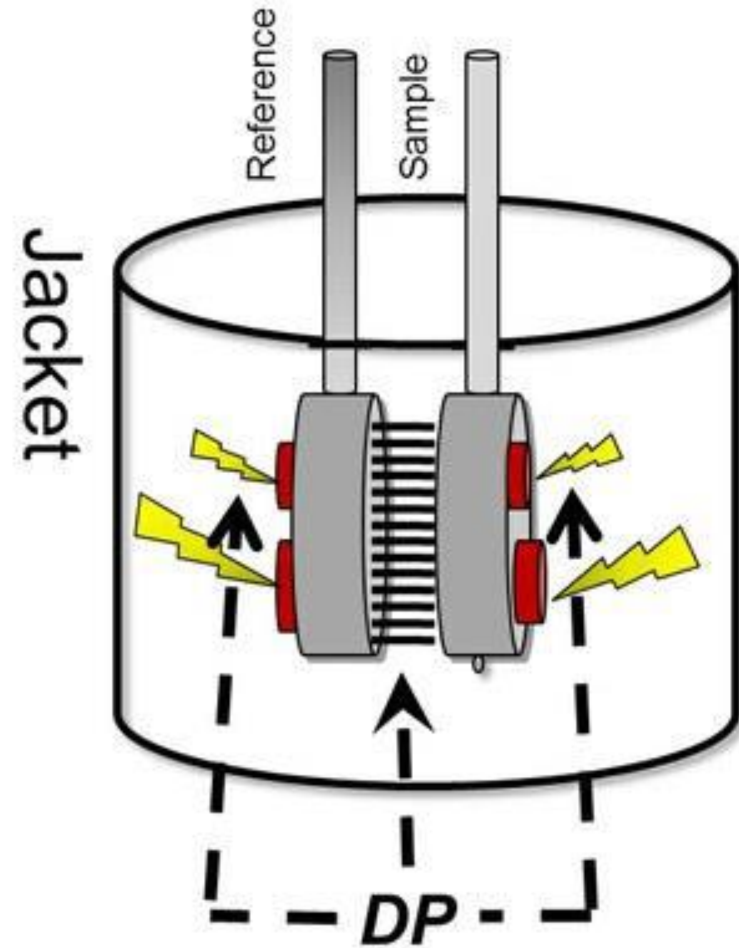
# DSC = Diferential Scanning Calorimetry

Sample cell (sample) and reference cell (buffer) are heated/cooled down at the same rate

Sample absorbs/release part of the energy causing a temperature difference between the sample and reference cell

DSC machine measures the energy needed to equalize the temperature

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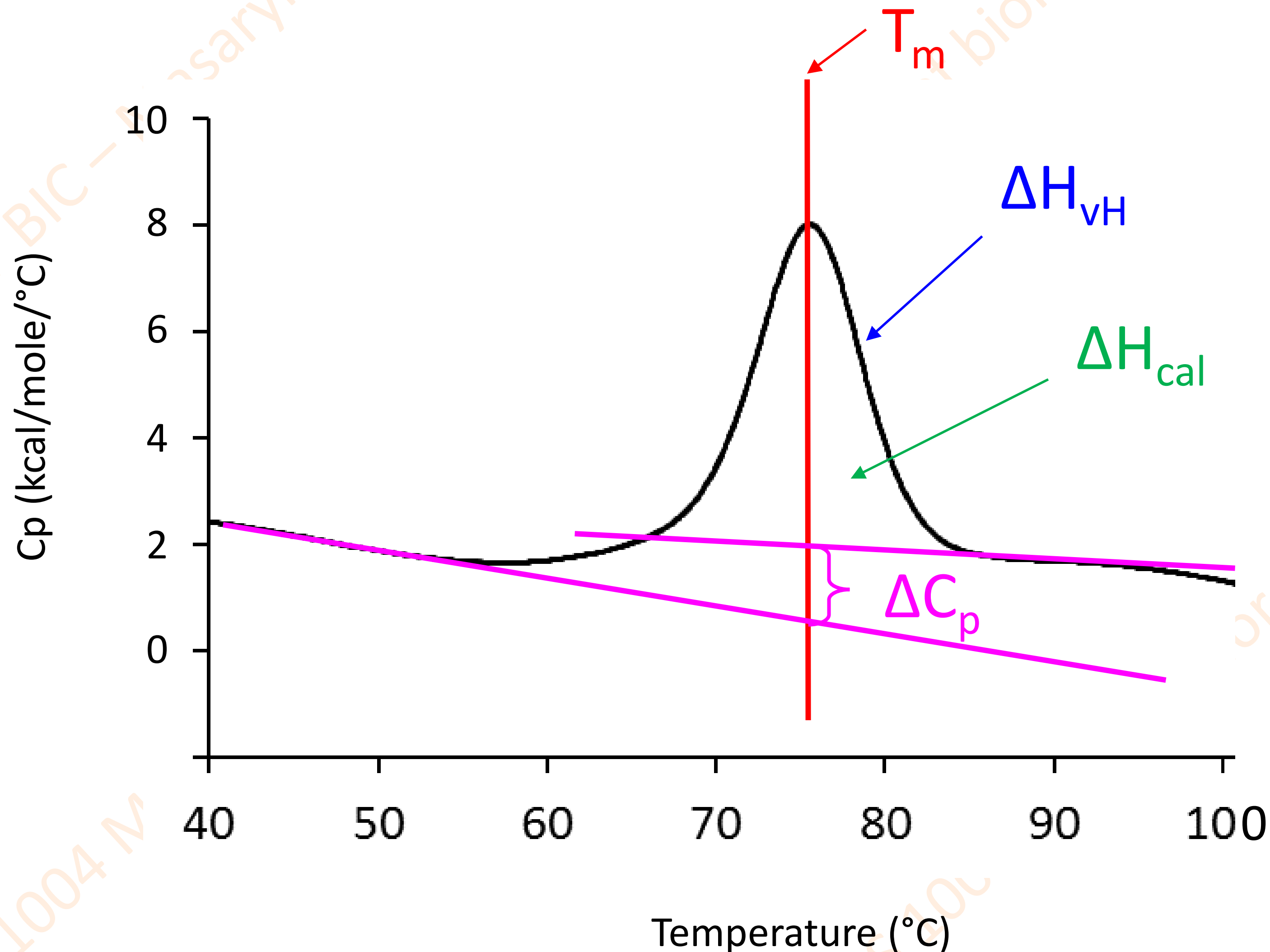
# DSC = Diferential Scanning Calorimetry




**$T_m$**
the peak of the transition

**$\Delta C_p$**
change of heat capacity folded-unfolded sample
Difference between two baselines

**$\Delta H_{cal}$**
Area of the peak (integration)

**$\Delta H_{vH}$**
The slope of the peak

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# DSC = Diferential Scanning Calorimetry

$\Delta H$ can be determined in two ways:

- **Directly** by calorimetric measurement – area under the peak – $\Delta H_{cal}$
- **Indirectly** by measureing the temperature dependance of the eqilibrium constant – van`t Hoff method, the slope of the peak, $\Delta H_{vH}$

If the difference between $\Delta H_{cal}$ and $\Delta H_{vH}$ is observed, it indicates that the reaction is more complicated – presence of intermediate state

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# DSC machines

## VP-DSC

1 sample at a time
0.8 ml sample at 0.1-2 mg/ml
Identical buffer in reference cell necessary (dialyses, lyophylization)
Degasing required

## Auto PEAQ-DSC

Automated version (up to 282 samples in a row)
0.2 ml sample at 0.1-2 mg/ml
Identical buffer in reference cell necessary (dialyses, lyophylization)

# DSC = Diferential Scanning Calorimetry

**Pros:**

- Direct measurement of thermodynamics
- Label free
- Gold standard for $T_m$ measurements
- Suitable for proteins, nuclei acids, lipids, polymers…

**Cons:**

- Time consuming
- High sample consumption

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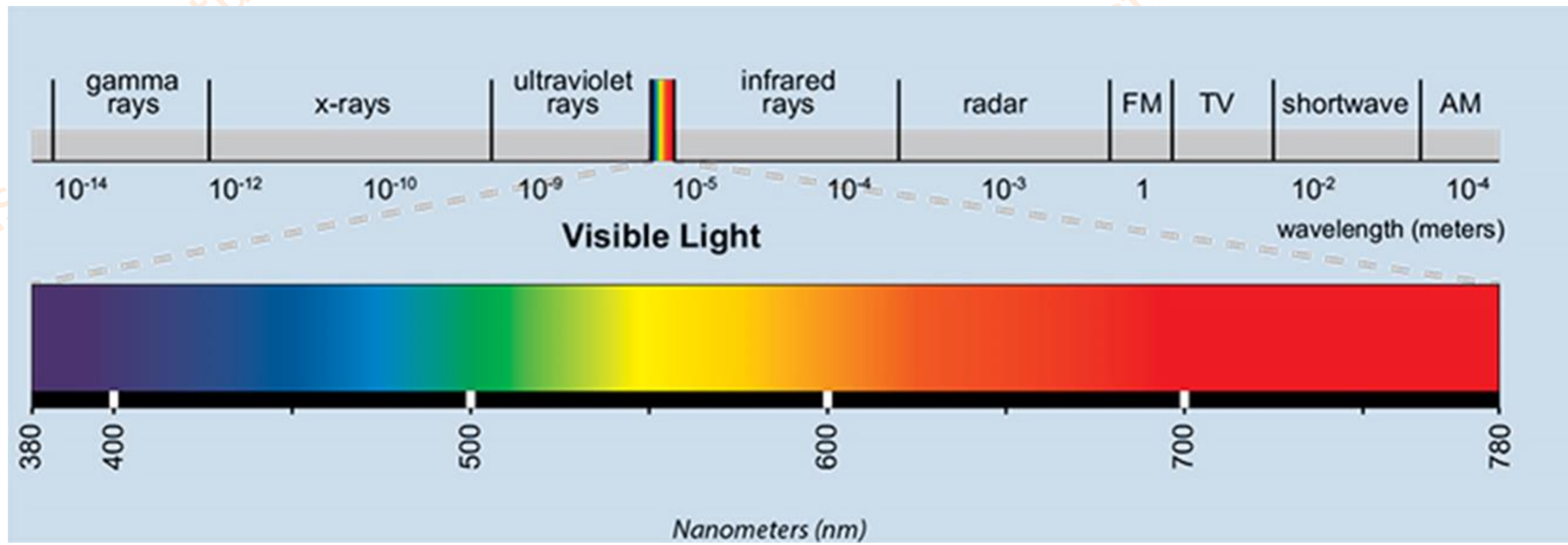
# Fluorescence

It is a physical phenomenon in which "light" is emitted by a substance that has previously absorbed electromagnetic radiation

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# TSA = Thermal Shift Assay

Also known as differential scanning fluorimetry ( = DSF)

High-throughput (96 well plates)

No specialized machine – uses termocycler for RT-PCR

Measures changes of fluorescence of the sample in temperature gradient

- Commercial dyes
- GFP-tag

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# TSA = Thermal Shift Assay

Commercial dyes (e.g. SYPRO Orange, bis-ANS, Nile Red):

Provide fluorescent signal only when they interact with hydrophobic residues of the unfolded protein

**Limitations**:
- Target protein do not have significant hydrophobic patches on the surface
- The target protein is folded at the begginning of experiment
- Dye do not bind to target protein
- Dye do not react with experimental buffer

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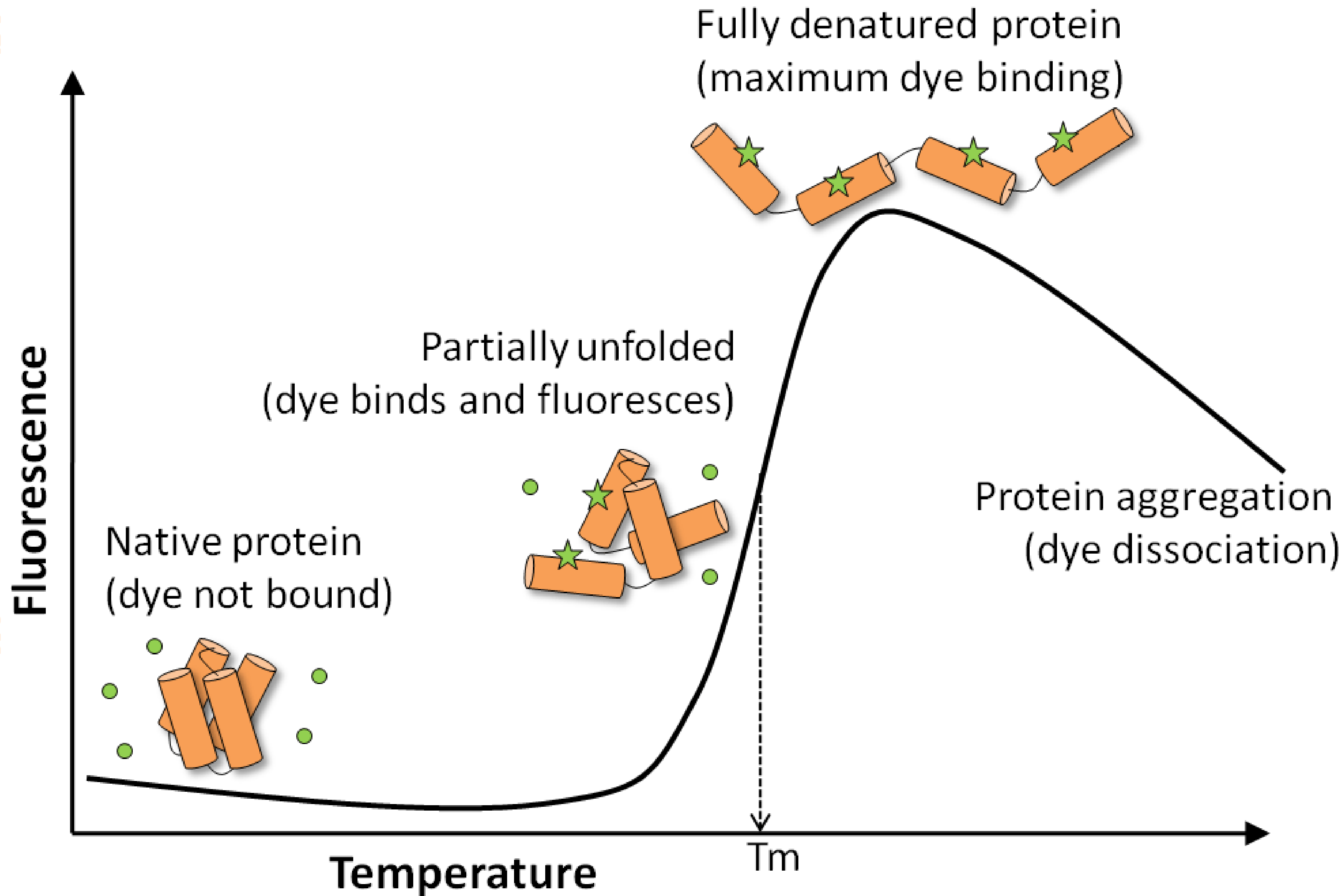
# TSA = Thermal Shift Assay

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# TSA = Thermal Shift Assay

GFP-tag:

GFP signal changes with its close environment, reports the unfolding of target protein

**Limitations**:
- $T_m$ of GFP is around 75 °C – only usable for less stable proteins
- Potential changes in conformation or oligomeric state of target protein after adding a GFP-tag

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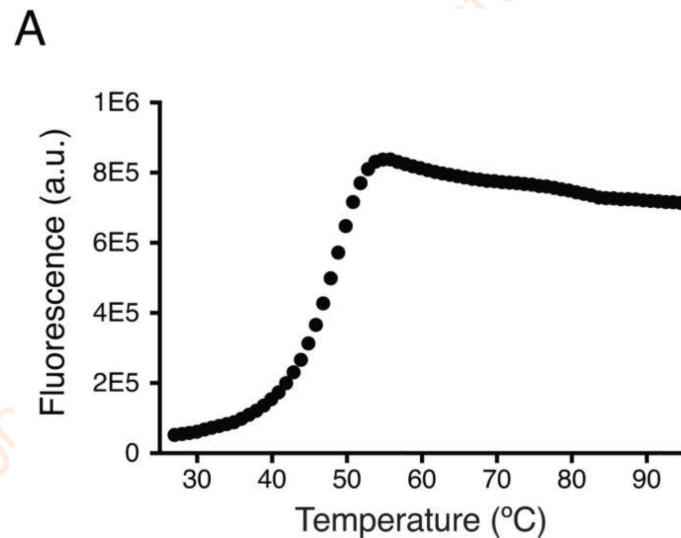
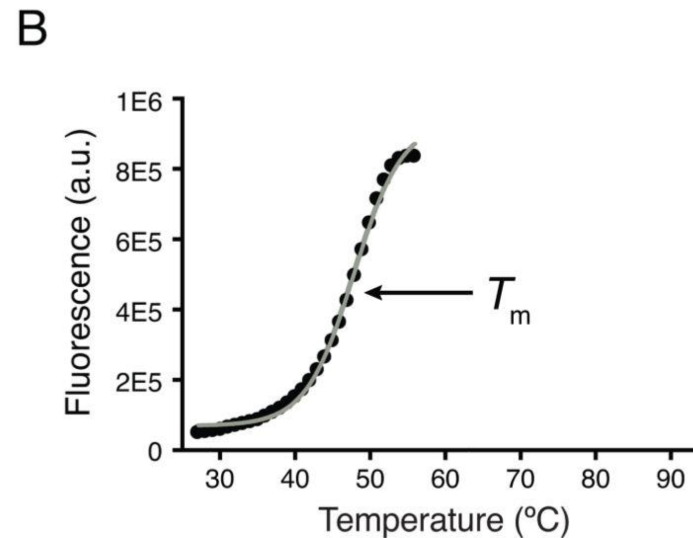
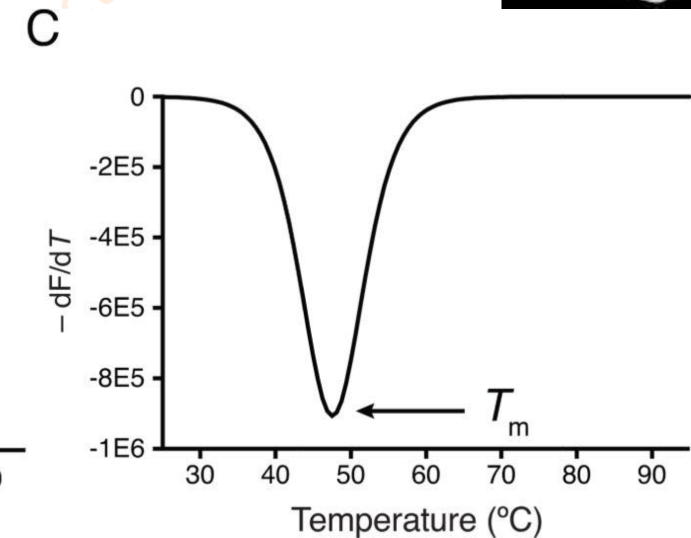
# TSA in practice

Protein and dye incubated in 96 well plates

Changes in fluorescence monitored




doi:10.1002/0471140864.ps2809s79

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# TSA = Thermal Shift Assay

**Pros:**

- Quick
- High-throughput
- Excelent for sample comparison
- Affordable instrumentation

**Cons:**

- Needs dye
- Usage of GFP-tag limited
- Data analysis

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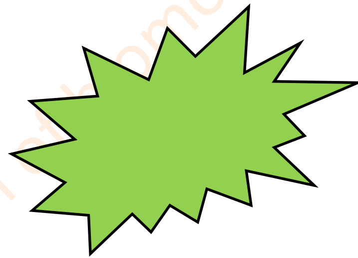
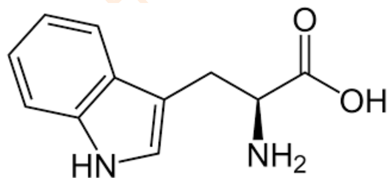
# Intrinsic fluorescence of proteins

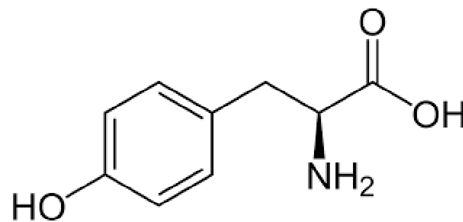


fluorophore

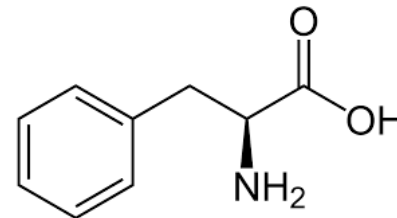
Tryptophan (Trp, W)




Tyrosine (Tyr, Y)




Phenylalanine (Phe, F)




Aromatic amino acids

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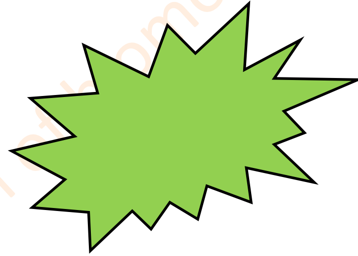
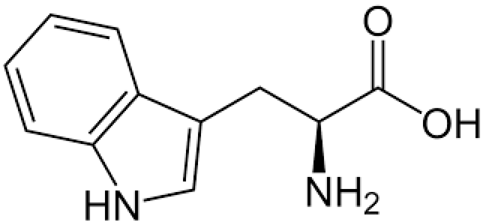
# Intrinsic fluorescence of proteins

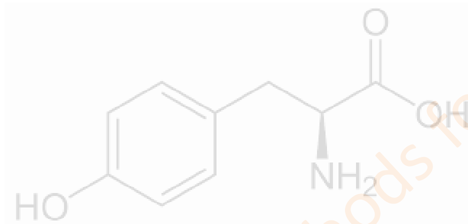


fluorophore

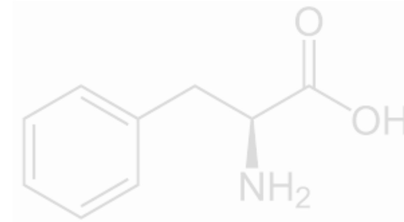
**Tryptophan (Trp, W)**




Tyrosine (Tyr, Y)




Phenylalanine (Phe, F)




Aromatic amino acids

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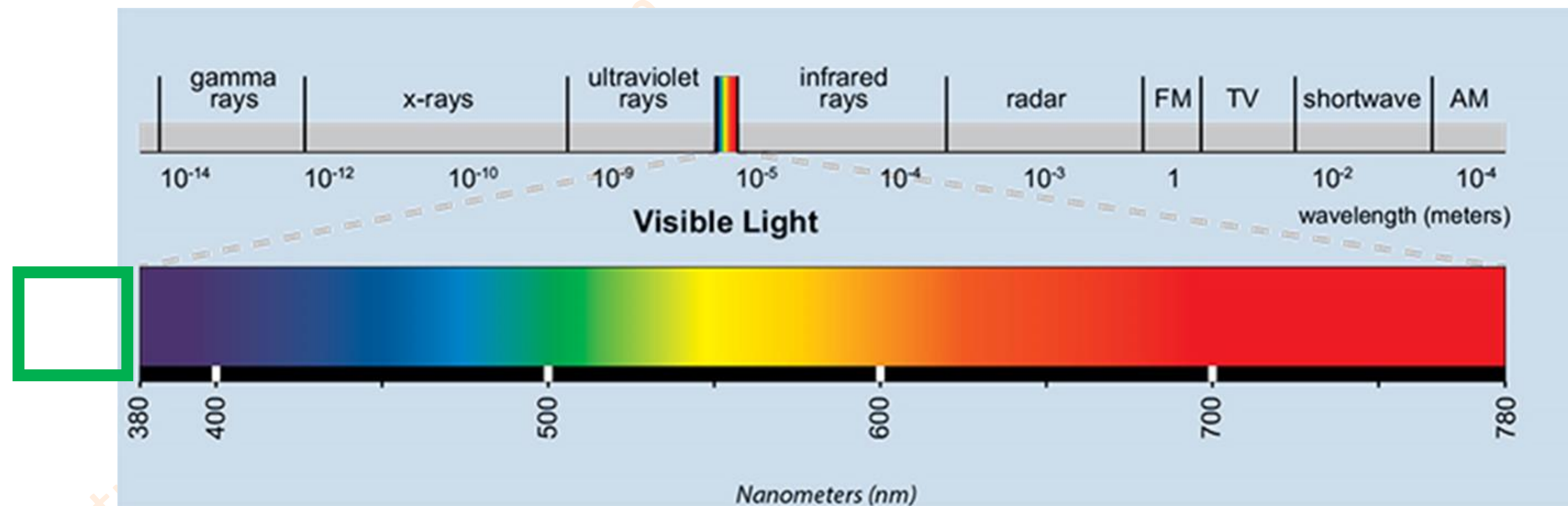
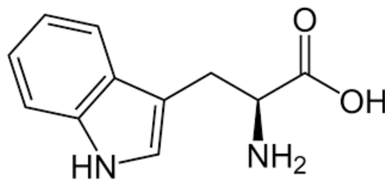
# Intrinsic fluorescence of proteins

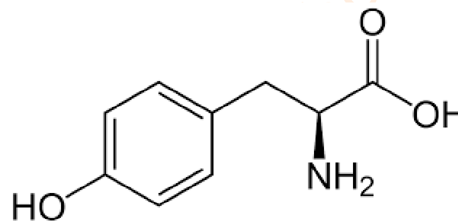
in UV region (λ = 300-360 nm)



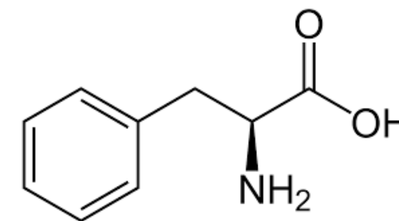

Tryptophan (Trp, W)




Tyrosine (Tyr, Y)




Phenylalanine (Phe, F)

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# nanoDSF = nano Diferential Scanning Fluorimetry

Measures changes of intrinsic fluorescence of the sample in temperature gradient

High-throughput (48 or 96 samples in 1 run)

Low sample consumption (10 µl)

Ideal for optimal condition screening

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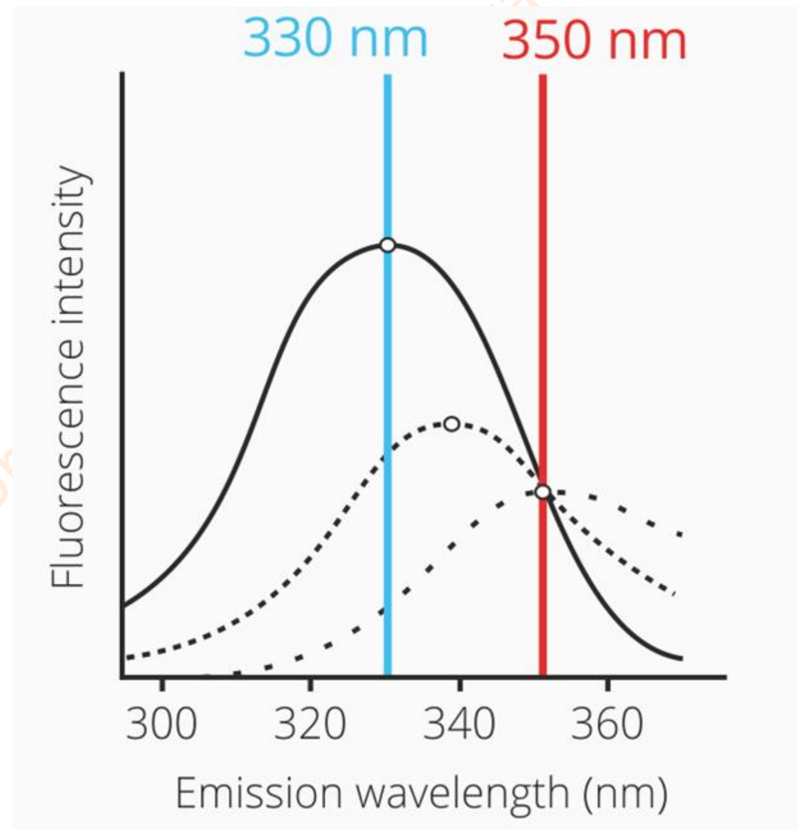
# nanoDSF

Intrinsic fluorescence of proteins (UV region, λ = 300-360 nm) is changing according to the local environment




——— hydrophobic

maximum at 330 nm

- - - - - hydrophilic

maximum at 350 nm

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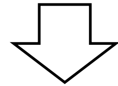
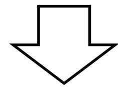
# nanoDSF

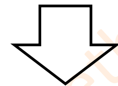
Aromatic aminoacids (W, Y, F) are hydrophobic and are typicaly located inside the folded protein



With increasing temperature the protein is unfolded



W, Y, F are exposed on the protein surface



Changes in fluorescence

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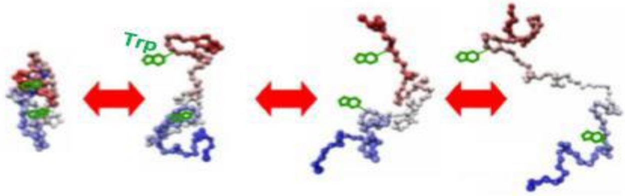
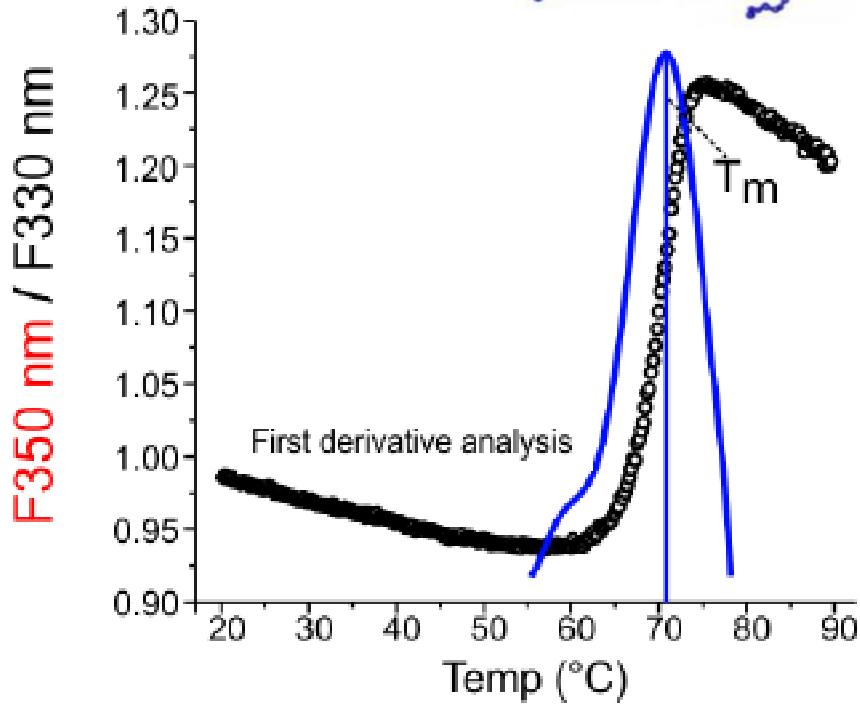
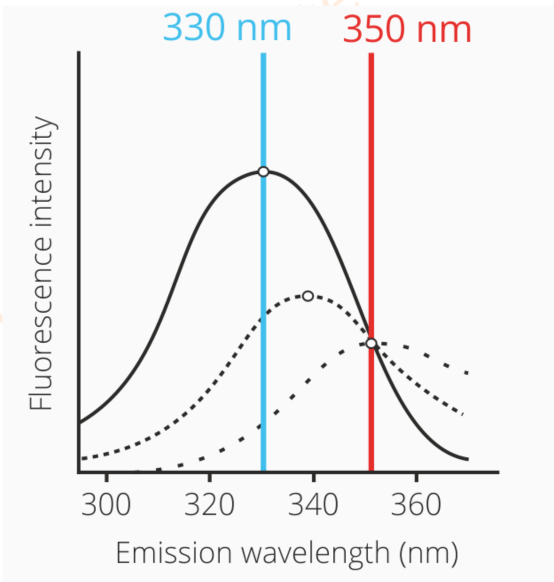
# nanoDSF





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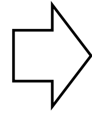
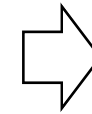
# nanoDSF in practice

Protein in buffers  Put into a capillary  48 samples

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# nanoDSF in practice

Design of experiment:

Temperature gradient 20 – 110 °C

Heating rate 1 °C/min

| | |
|---|---|
| quicker | higher Tm |
| slower | lower Tm |

Prometheus

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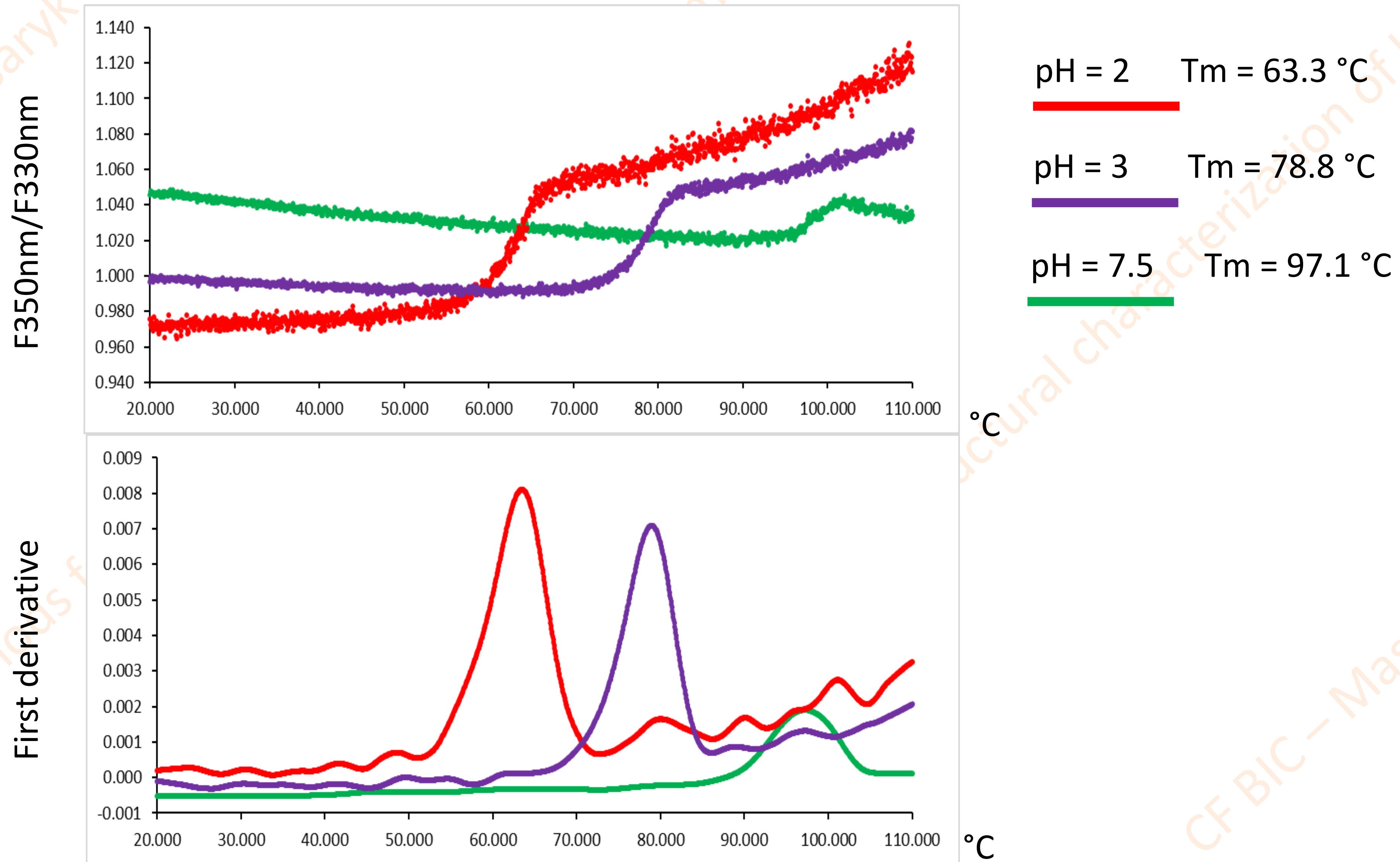
# nanoDSF in practice

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# nanoDSF in practice

| °C | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 20 mM Tris, 150 mM NaCl pH 7.5 | 50 mM maleate pH 2.0 | 100 mM glycine pH 3.0 | 100 mM formate pH 4.0 | 100 mM citrate pH 5.0 | 100 mM cacodylate pH 6.0 | 100 mM Hepes pH 7.0 | 100 mM bicine pH 8.0 | 100 mM CHES pH 9.0 | 50 mM borate pH 10.0 | 100 mM CAPS pH 11.0 | 100 mM phosphate pH 12.0 |
| | 97.1 | 63.3 | 78.7 | 93.0 | 99.4 | 95.2 | 96.9 | 90.5 | 86.3 | 75.2 | 64.3 | 56.7 |
| B | 100 mM acetate pH 4.0 | 100 mM acetate pH 4.5 | 100 mM acetate pH 5.0 | 100 mM MES pH 5.5 | 100 mM MES pH 6.0 | 100 mM MES pH 6.5 | 100 mM Na phosphate pH 7.0 | 100 mM K phosphate pH 7.5 | 100 mM Tris pH 8.0 | 100 mM Tris pH 8.5 | 100 mM glycine pH 9.0 | 100 mM glycine pH 9.5 |
| | 93.9 | 95.6 | 96.7 | 96.5 | 96.8 | 95.9 | 70.2 | 70.5 | 94.0 | 92.1 | 91.9 | 88.0 |
| C | 100 mM MES, 100 mM NaCl, pH 6.0 | 100 mM MES, 200 mM NaCl, pH 6.0 | 100 mM MES, 500 mM NaCl, pH 6.0 | 100 mM MES, 1000 mM NaCl, pH 6.0 | 100 mM Na phosphate, 100 mM NaCl, pH 7.0 | 100 mM Na phosphate, 200 mM NaCl, pH 7.0 | 100 mM Na phosphate, 500 mM NaCl, pH 7.0 | 100 mM Na phosphate, 1000 mM NaCl, pH 7.0 | 100 mM Tris, 100 mM NaCl, pH 8.0 | 100 mM Tris, 200 mM NaCl, pH 8.0 | 100 mM Tris, 500 mM NaCl, pH 8.0 | 100 mM Tris, 1000 mM NaCl, pH 8.0 |
| | -.- | 97.1 | 96.9 | -.- | -.- | -.- | -.- | 93.7 | 93.3 | 93.0 | 93.1 | 94.9 |
| D | 20 mM Tris, 150 mM NaCl, 100 uM CaCl2, pH 7.5 | 10 mM HEPES, 150 mM NaCl, 0.05% Tween pH 7.5 | 12 mM PBS, 130 mM NaCl, 2.7 mM KCl, pH 7.5 | 12mM PBS, 0.05% Tween 20, pH 7.5 | 200 mM imidazole, pH 7.5 | 0.05% Tween 20 | 5% glycerol | 5 mM bME | 5% DMSO | 5% trehalose | 20 mM arginine, 20 mM glutamine | 5 mM EDTA |
| | 96.1 | 95.2 | -.- | -.- | 94.2 | 96.4 | 98.2 | 96.2 | 97.8 | 97.6 | 95.0 | 95.3 |

# nanoDSF

## Pros:

- Quick
- High-throughput
- Low sample consumption (10 µl)
- Low concentration (0.1 – 1 mg/ml)
- No labelling
- Excelent for sample comparison
- User friendly instrumentation

## Cons:

- Only for proteins
- W (Y, F) in sequence necessary
- Sensitive to capillary purity
- Delicate manipulation with capillaries

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# Applications

- Thermal stability determination
- Ligand screening
- Buffer optimization for purification and storage
- Optimization of crystallization conditions
- Batch to batch comparison

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# Comparison

| | DSC | TSA | nanoDSF |
|---|---|---|---|
| sample | proteins, nucleic acids, lipids, polymers | proteins | proteins |
| Sample consumption | high | low | low |
| High-throughput | no | yes | yes |
| Automation | yes | no | no |
| Enthalpy | yes | no | indirect |
| Fluorescent dye | no | yes | no |

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# Literature

Protein unfolding:
Konnermann L.: Protein unfolding and denaturants
doi: 10.1002/9780470015902.a00030004.pub2

DSC:
Chiu M.H., Prenner E.J.: Differential scanning calorimetry: An invaluable tool for a detailed thermodynamic characterization of macromolecules and their interactions.
doi: 10.4103/0975-7406.76463

nanoDSF:
Alexander C.G, Wanner R., Johnson C.M., et al.: Novel microscale approaches for easy, rapid determination of protein stability in academic and commercial settings
doi: 10.1016/j.bbapap.2014.09.016

TSA:
Gao K.,Oerlemans R., Groves M.R.: Theory and application of differential scanning fluorimetry in early-stage of drug discovery.
doi: 10.1007/s12551-020-00619-2.

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# Biomolecular Interactions and Crystallography Core Facility

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