

3. *In vitro* Sumoylation assay

I. Process workflow

- a. Preparation of mixtures
- b. Incubation
- c. SDS-PAGE
- d. Staining and destaining

II. Motivation

The students will practice the basics of post-translational modifications performed *in vitro*. In this case the protein will be covalently modified by the SUMO protein. Such post-translationally modified proteins can be then studied in comparison to their unmodified counterparts to elucidate the biological significance of the modification.

III. Theoretical background

Post-translational modifications play an essential role in all aspect of cellular metabolism. SUMO protein is a member of the ubiquitin-like protein (Ubl) family whose founding member is ubiquitin. The process where SUMO protein is covalently attached to other proteins in cells to modify their functions is called SUMOylation.

The mechanism of this pathway is very close to ubiquitylation but it utilizes a different set of enzymes. The C-terminal carboxyl group of SUMO is covalently linked to AMP in an ATP-dependent activation step catalyzed by an E1-activating enzyme, which is a heterodimer consisting of SAE1 and SAE2 subunits (or Aos1 and Uba2 in budding yeast). Activated SUMO is then transferred to an E2-conjugating enzyme. In contrast to ubiquitylation, there is only a single known SUMO E2 enzyme – Ubc9. Ubc9 is highly conserved from yeast to humans and plays an essential role in cell cycle regulation. In the ubiquitin system, E3 ligases are necessary for ubiquitin attachment to specific target proteins. However, SUMO conjugation is not strictly dependent on E3 catalysis. A typical SUMO acceptor site consists of a specific sequence – the ψ KxE motif, where ψ is a large hydrophobic amino acid (e.g. isoleucine, valin), K is the target lysine, x is any residue followed by glutamic acid residue E (Meulmeester, 2008).

However, it must be taken into consideration that not all proteins with this motif are SUMOylated. On the other hand, the SUMOylated protein does not have to contain a consensus SUMO acceptor site. Thus, other factors such as subcellular localization and E3 ligases appear to modulate the efficiency and specificity of SUMOylation. Also in contrast to ubiquitylation, SUMO modification does not tag proteins for proteosomal degradation, but rather alters protein-protein interactions, subcellular nuclear localization, protein-DNA interaction and control of protein stability (SUMO can also act as an antagonist of ubiquitin)(Hay, 2005).

In this experiment, you will check the SUMOylation of Rad52 protein using *in vitro* SUMOylation system. Rad52 is a DNA binding protein which is essential for the error-free repair of DNA double-strand breaks by homologous recombination. It mediates the exchange of the recombination factor RPA associated with single-stranded DNA by Rad51, resulting in the assembly of the presynaptic filament. Recently, it has been reported that upon DNA damage Rad52 is modified by the small ubiquitin-like modifier (SUMO) protein which shelters Rad52 against proteosomal degradation and regulates recombinational repair at the ribosomal gene locus (Sacher, 2006; Symington, 2002).

IV. Design of the experiment

Material:

5x reaction buffer S3 (50 mM HEPES, 50 mM MgCl₂, 0.5 mM DTT)

ATP (c = 100 mM)

10% SDS-polyacrylamide gel

SDS Laemmli buffer (62.5 mM Tris-HCl, 2% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.002% bromphenol blue)

Coomassie Brilliant Blue staining solution

10xSDS buffer (151.38 g Tris, 814.61 g glycine, 50 g SDS, dissolve in 5 L of dH₂O)

Destain (25% methanol, 12.5% acetic acid)

Sterile H₂O

Proteins:

GST-tagged Aos1/Uba2 heterodimer (yeast E1 enzyme)

(His)₆-tagged Ubc9 (yeast E2 enzyme)

(His)₆-tagged Smt3 (yeast SUMO protein)

Rad52 (target protein)

Protocol:

a. Preparation of mixtures

1) Prepare the samples according to the following table. Add the ATP at the end to start the reaction, after you have pipetted all the other components. Mix and spin shortly.

	5xS	E1	E2	Smt3	Rad52	H ₂ O	ATP
nr. 1	4.0	0.7	1.0	1.5			-
nr. 2	4.0	0.7	1.0	1.5			0.5
nr. 3	4.0	0.7	1.0	1.5			0.5

Total reaction volume = 20 µL

b. Incubation

2) Incubate the samples for 2 hours at 30 °C.

3) Stop the reaction by adding 30 µL of SDS-Laemmli Page

c. SDS-PAGE

4) Place the SDS gel in the vertical electrophoresis unit filled with 1x SDS buffer

5) Boil the samples 2 min in hot water and load 10 µL of samples on the gel.

6) Run the gel for 45 min at 200 V.

7) After the run is over, remove the gel sandwich from the electrophoresis unit. Separate both glasses and place the gel in the Coomassie staining solution.

d. Staining and destaining

8) Incubate in the Coomassie staining solution for 10 min.

9) Transfer the gel to the box containing destain solution.

V. References

1. Meulmeester, E., and Melchior, F., *Cell biology: SUMO*. Nature, 2008. **452**(7188): p. 709-11.
2. Hay, R. T., *SUMO: a history of modification*. Mol Cell, 2005. **18**(1): p. 1-12.
3. Sacher, M., Pfander, B., Hoege, C., and Jentsch, S., *Control of Rad52 recombination activity by double-strand break-induced SUMO modification*. Nat Cell Biol, 2006. **8**(11): p. 1284-90.
4. Symington, L. S., *Role of RAD52 epistasis group genes in homologous recombination and double-strand break repair*. Microbiol Mol Biol Rev, 2002. **66**(4): p. 630-70, table of contents.

VI. Question

Approximately what portion of Rad52 protein was sumoylated in the assay?

