Large-scale in vitro PCNA ubiquitinylation by Rad18/Rad6

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INTRODUCTION

PCNA is monoubiquitinylated on lysine K164 by the E2/E3 enzymes Rad6/Rad18. This modification provides an essential first step in DNA damage tolerance pathways by signaling for recruitment of Y-family polymerases capable of replication past sites of DNA damage. We have reconstituted this ubiquitinylation reaction *in vitro* using recombinantly expressed E1, Rad6/Rad18, PCNA and ubiquitin proteins on a µM scale. The modified product is specifically ubiquitinylated on K164 and is suitable for biochemical experiments such as binding studies or activity assays for deubiquitinylation enzymes.

MATERIALS

Reagent

Suggested ratios in 100µL reactions

Reagent	Stock Solutions	Suggested Volume	Final Concentration
Rad18/Rad6	3.5mg/mL = 50µM	20µL	10µM
PCNA	9.2mg/mL = 105µM	3µL	3.2µM
Ub	11.0mg/mL = 1.3mM	10µL	130µM
E1 (wheat)	2.0mg/mL = 17µM	10µL	1.7µM
ATP/Mg^{2+}	100mM	10µL	10mM
DTT	100mM	0.1-1µL	0.1-1mM
		Add to 100µL	

Add to 100µL with 25mM Tris buffer pH 8.0 (not lower – needs to be slightly basic for the reaction to occurs) and 150mM NaCl, 5µM βME

METHODS

1. Pipette E1, buffer, ATP/Mg, DTT and ubiquitin together first. This will upload the E1.
2. Pipette Rad18/Rad6 and PCNA together. This will pre-bind the target and minimise autoubiquitination.
3. Combine the two mixes.
4. Incubate for up to 4 hours at 30°C or overnight at 18°C.
5. Rad18 is massively autoubiquitinated, so the ubiquitin needs to be in large excess. Some loose ubiquitin chains are also formed.

BIBLIOGRAPHY

Notenboom V, Hibbert RG, van Rossum-Fikkert SE, Olsen JV, Mann M, Sixma TK. Functional characterization of Rad18 domains for Rad6, ubiquitin, DNA binding and PCNA modification. ***Nucleic Acids Res.*** 2007; 35:5819-30.