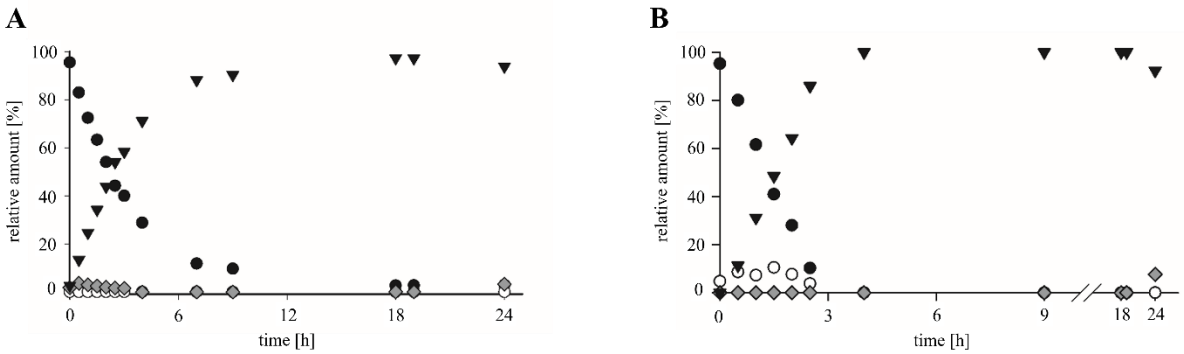
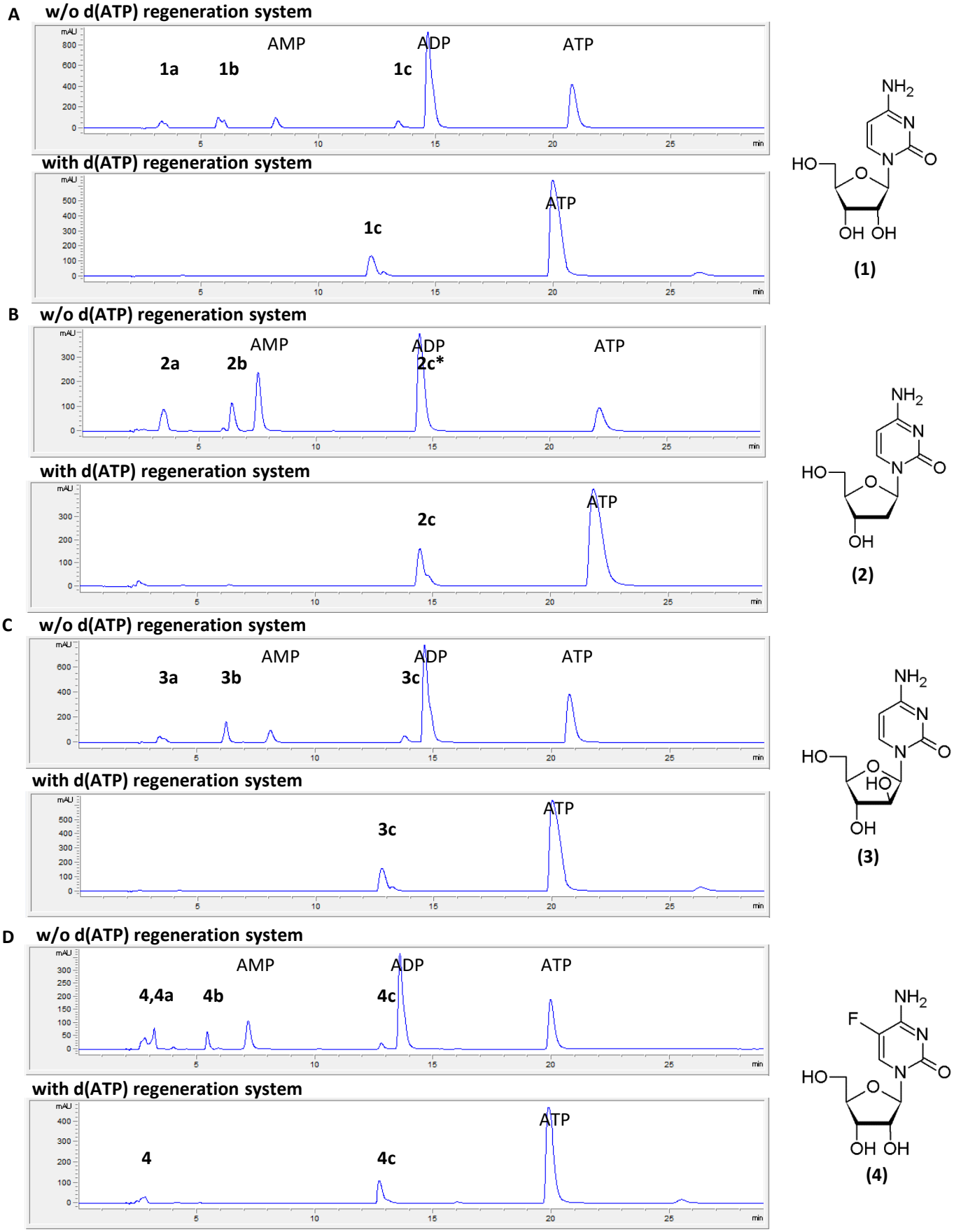
Supplementary Material



Supplementary Figure 1. Time course for the synthesis of natural (**A**, **1a-c**) and modified nucleotides (**B**, **3a-c**) in an one-pot multi-enzyme cascade reaction. The formation of NMP ○, NDP ◆ and NTP ▼was analyzed over 24 h using the natural nucleoside cytidine (**1**, **A**) and sugar-modified nucleoside analog arabinosylcytosine (**3**, **B**) as substrates (●). The enzyme combination *Dm*dNK / UMP-CMPK / NDPK was applied. The first datapoint was taken 30 s after reaction initiation. Experimental conditions: 1 mM substrate, 3.6 mM phosphate donor, 70 mM Tris HCl pH 7.6, 5 mM $MgCl_2$, 0.016−0.02 mg/mL each cascade enzyme, 5 mM phosphoenolpyruvate and 0.17 mg/mL pyruvate kinase, 37°C.

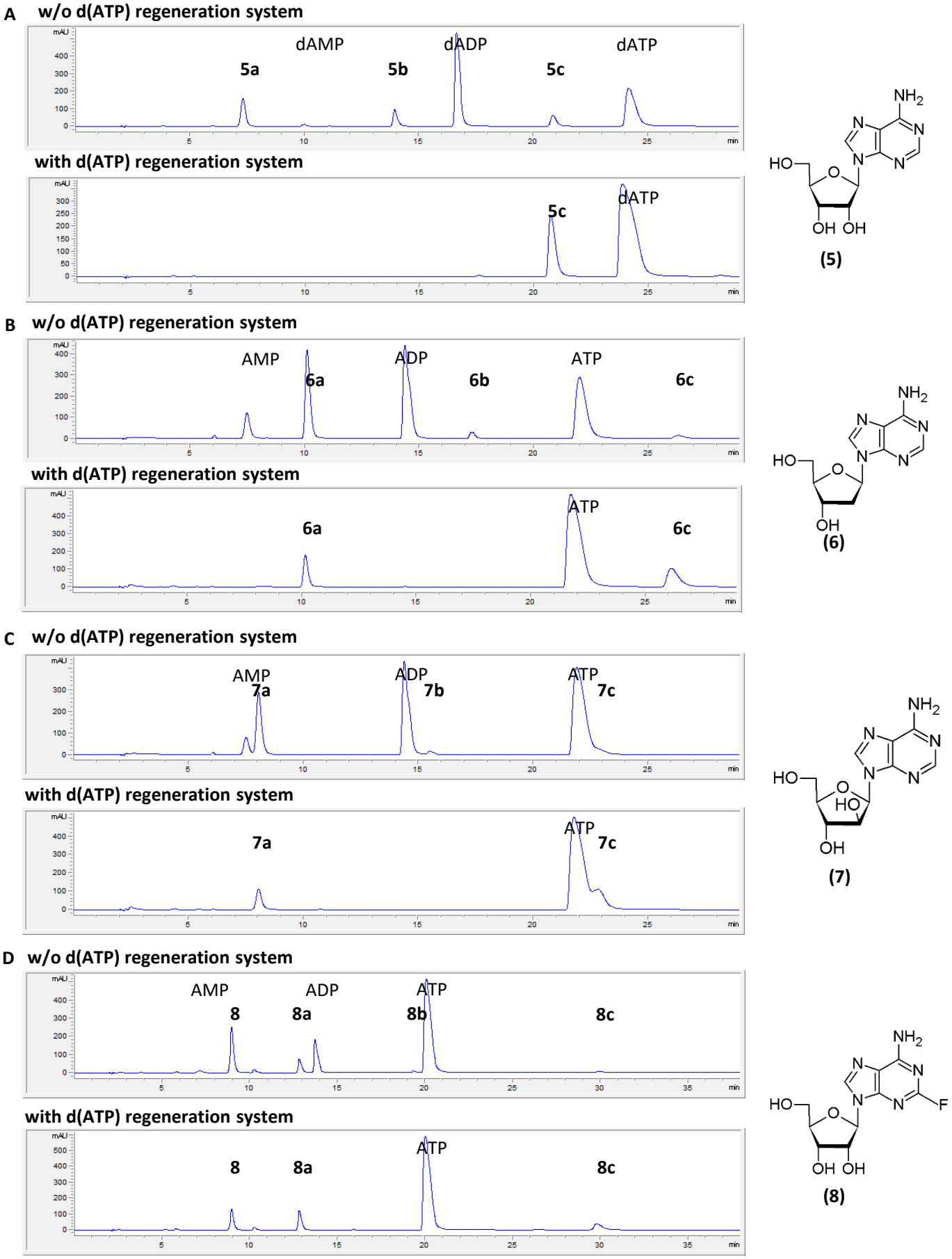
Table SI. Enzymes and enzyme concentrations applied for the synthesis of natural and modified NTPs in the absence and presence of a (d)ATP recycling system.

Compound	NK		NMPK		NDPK	
	Enzyme	Conc. [mg/ml]	Enzyme	Conc. [mg/ml]	Enzyme	Conc. [mg/ml]
1c	*Dm*dNK	0.016	UMP-CMPK	0.016	NDPK	0.016
2c	*Dm*dNK	0.016	UMP-CMPK	0.016	NDPK	0.016
3c	*Dm*dNK	0.016	UMP-CMPK	0.016	NDPK	0.016
4c	*Dm*dNK	0.020	UMP-CMPK	0.020	NDPK	0.020
5c	AK	0.020	GMPK	0.020	NDPK	0.020
6c	*Dm*dNK	0.016	UMP-CMPK	0.016	NDPK	0.016
7c	*Dm*dNK	0.016	UMP-CMPK	0.016	NDPK	0.016
8c	AK	0.020	AMPK	0.020	NDPK	0.020



Supplementary Figure 2. HPLC chromatograms obtained during the synthesis of natural and modified pyrimidine NTPs 1c (A), 2c (B), 3c (C), 4c (D) in the absence and presence of the (d)ATP regeneration system.

) Compound **2 top figure (without ATP regeneration): The dCTP (2c) peak overlays with ADP peak and therefore cannot be evaluated. Therefore, the dCTP amount was calculated from dCMP and dCDP amounts. With ATP regeneration (lower figure) there was no ADP present judged by the UV absorption spectrum of the respective peak 2c.*



Supplementary Figure 3. HPLC chromatograms obtained during the synthesis of natural and modified purine NTPs 5c (A), 6c (B), 7c (C), 8c (D) in the absence and presence of the (d)ATP regeneration system.