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Cell-free expression of human blood group A and B glycosyltransferases

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Cell-Free Protein Synthesis

Our initial NMR studies on human blood group A and B glycosyltransferases (GTA and GTB) have shown that classical assignment procedures using e.g. 3D triple resonance spectra fail. In order to allow for combinatorial labeling approaches we had to establish a cell free expression protocol instead. Fig. 1 summarizes components required for cell-free protein synthesis (T7 RNA Polymerase, S30-extract containing more than 200 proteins and ribosomes for translation, Mg^{2+} and K^{+} ions, p*IVEX* or p*ET*-vector coding for the target protein, ATP, GTP, UTP, CTP, amino acids, tRNA, Creatine kinase/phosphocreatine for energy recycling, PEG 8000 to mimic the viscosity of cytosol and more compounds and salts [1, 2].

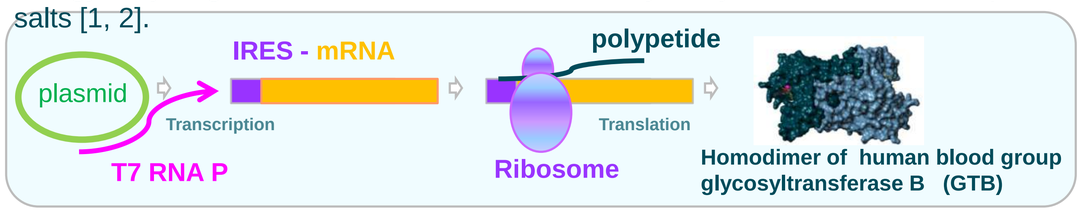

Fig. 1: Coupled system for the cell-free protein synthesis with transcription and translation

T7 RNA Polymerase

T7 RNA P was expressed in 1 L M9-minimal medium [1] using the vector pAR1219 in BL21 (DE3). T7 RNA P has to be protected from degradation by proteases during purification. Long time storage is achieved with 100 mM NaCl and 50% glycerol at -80° C. The activity of the T7 RNA P depends strongly on Mg^{2+}. 100 mM NaCl is an inhibitor of T7 RNA P and NaCl also inhibits the cell-free protein synthesis at >50 mM.
We purified 32 mg (7 mg/ml) protein with a purity of T7 RNA P of 90%.

S30 - Extract

An A19 strain (RNA and amino acid stabilizing mutations) was used to prepare S30-extract [1, 2] with two different media in a 10 L fermenter: 2xYTPG medium [2] and incomplete rich medium [1] , a minimal medium supplemented with 0.1% NH_4Cl and 4 mM $MgSO_4$. Only 50% of our S30-extracts prepared in flask cultures were active. Only with a fermenter culture we got enough S30-extract for testing. Essential for an active S30-extract are: a short lag-phase and generation time and fast cooling before harvesting the cell pellet.

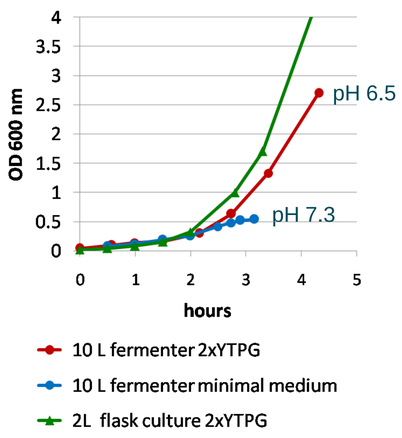

Fig. 2: Growth curve A19 in fermenter and flask culture

	10 L fermenter		1 L flask culture	
Medium	2xYTPG	Minimal medium	2xYTPG	Minimal medium
Harvest OD_{600}	3	0.54	1.2	0.6
pellet	66 g	13 g	2.5 g	0.6 g
S30-extract	26 ml	3.1 ml	0.7 ml	1 ml
Mg^{2+} f.c.	10.3 mM	10.3 mM	15 mM	16 mM

	10 L fermenter		1 L flask culture	
Quality control (25-40 mg/ml)	2xYTPG	Minimal medium	2xYTPG	Minimal medium
Protein Bradford/BSA	19 mg/ml	23 mg/ml	(250 mg/ml)	16 mg/ml

Tab. 1: Quality data S30-extracts
The generation time of all cultures is 30-40 min. Important is a high protein concentration >25 mg/ml.

The batch-to-batch activity of S30-extract preparations also depends on the following steps: **1)** Fast cell lysis; **2)** Run-off to release endogenous mRNA from the ribosomes (45° C for 45 min., no preincubation mix, 400 mM NaCl); **3)** Separation of the 30S and 50S subunits from the 70S ribosomes (not active in cell-free protein synthesis) by high speed centrifugation steps; **4)** Cytosol matrix is exchanged against S30-buffer by dialysis (50 mM Tris/acetate, pH 8.2 (RT), 14 mM $Mg(OAc)_2$, 60 mM KOAc, 0.5 mM DTT); **5)** Endogenous amino acids have to be removed completely for stable isotope labeling of proteins for NMR measurements. Several dialysis steps with buffer exchange are sufficient.
After a last centrifugation step at 30.000xg the S30-extract is shock frozen in liquid nitrogen and stored at -80° C for max. one year.

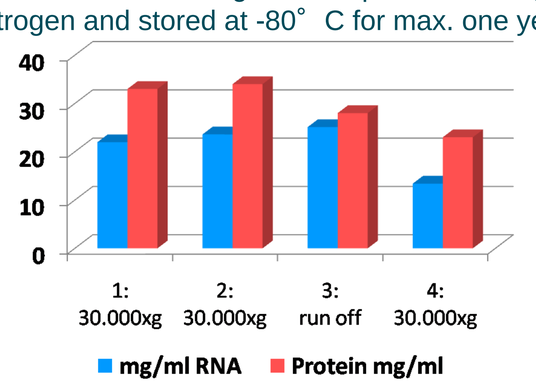

Fig. 3: Protein concentration during S30-extract preparation
From cell lysis to final S30-extract only 66 % of the total protein concentration has been retained. In any case, it is important to start with high protein concentration in the lysis.

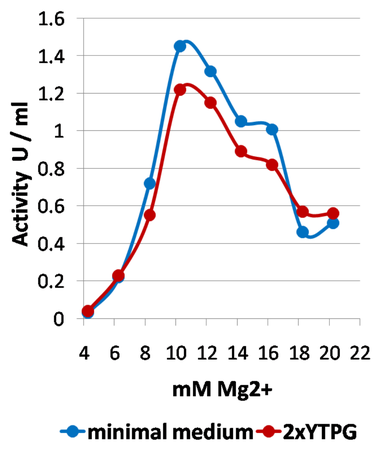

Fig. 4: Mg-Titration of S30-extract
Usually the Mg-concentration is >15 mM. Probably this reflects the low protein concentration in both S30-extracts.

Results: 50 µl Batch Reaction

Cell-free protein synthesis in 50 µl batch reactions: 30% S30-extract, 120 µg/ml T7 RNA P, 27 mM NH4OAc, 10.3 mM Mg(OAc)2, 100 mM KOAc, 100 mM Hepes/KOH pH 7.5, 250 µg/ml creatine kinase, 52 mM phosphocreatine di-Tris salt, 2 mM amino acids, 2 mM (Arg, Cys, Trp, Met, Asp, Glu)-mix, 2% PEG 8000, 1.2 mM ATP, 0.8 mM each CTP, GTP, UTP, 640 µM cAMP, 68 µM folinic acid, 180 µg/ml tRNA, 1.7 mM DTT, 20 µg/ml plasmid, 0.1 U/ml RNasin, 1x protease inhibitor. The reaction was performed in 200 µl tubes for 20 hours at 30° C and 600 rpm (highest activity of GTB after 20 hrs) [1, 2].

	10 L Fermenter		2L flask culture	
Medium	2xYTPG	Minimal medium	2xYTPG	Minimal medium
2 mM amino acids	1.2 U/ml	1.4 U/ml	0.2 U/ml	0.5 U/ml
	1300 x10^3 DPM	1500 x10^3 DPM	233 x10^3 DPM	567 x10^3 DPM
0 mM	48 DPM	65 DPM	138 x10^3 DPM	150 DPM

Tab. 2: Activity of GTB with S30-extracts from fermenter and flask cultures
Intensive dialysis seems to be sufficient to remove endogenous amino acids from S30-extracts. An ultimate opinion is only possible after the first labeling and NMR experiments.

	Vector 1 pIVEX-GTB	Vector 2 pIVEV-His_6-TEV-GTB
Activity assay	1 U/ml	0.3 U/ml
Insoluble GTB	40%	70%

Tab. 3: Vectors for GTB cell-free synthesis
The GTB activity with vector 2 was measured after TEV cleavage.

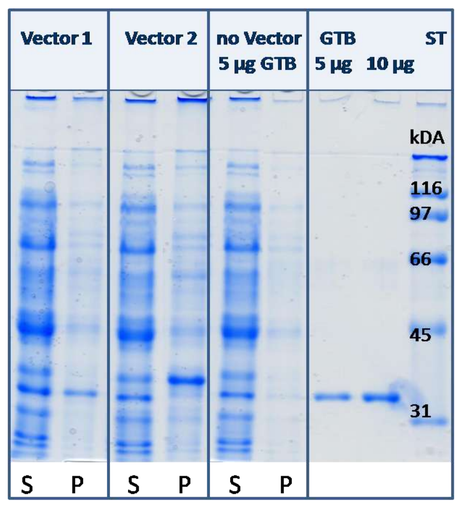

Fig. 5: SDS-PAGE of GTB batch reaction mix
The vectors are cause of different amounts of soluble S and insoluble P GTB. Reaction mix without plasmid and addition of GTB (20 hrs incubation). Reference: GTB

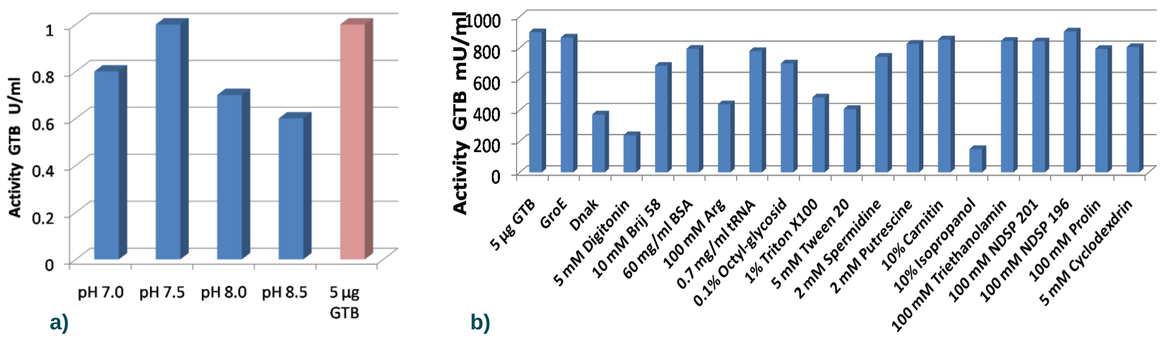

Fig. 6: Attempts to increase the amount of soluble GTB with chemical chaperons
It seems GTB has folding problems in the cell-free expression, especially with vector 2.
a) Purified GTB is stable during cell-free reaction and also the pH value of 7.5 of the reaction buffer Hepes/KOH is ideal. **b)** Only DnaK causes no insoluble GTB but low yield.

Results: 50 µl Continuous Exchange Reaction

The advantage of continuous exchange cell-free protein synthesis is continuous supply of new reagents, especially ATP, and removal of side products like phosphate, which inhibit protein synthesis. Each reaction mix contains S30-extract, T7 RNA P, plasmid, tRNA and is separated from the feeding mix via a dialysis membrane (MWCO 10 kDa). The ratio of reaction mix to feeding mix is about 1:18.

	Device 1	Device 2	Device 3	Device 4	Batch
Feeding mix	950 µl	750 µl	750 µl	950 µl	-
Reaction mix	50 µl	50 µl	50 µl	50 µl	50 µl
End volume	25 µl	40 µl	45 µl	10 µl	50 µl
GTB Activity	3.9 U/ml	3.68 U/ml	1.73 U/ml	3 U/ml	0.8 U/ml

Fig. 7: Comparison of the devices with vector 1
The self made device 2 has the highest activity of GTB with a good recovery of the reaction mix. Maybe device 4 reflects only the concentration of the reaction mixture in the membrane?

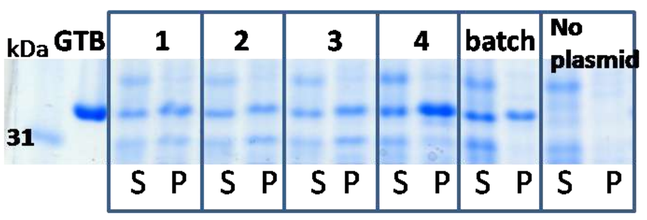

Fig. 8: SDS-PAGE of soluble and insoluble GTB in dependence of the device

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[1] Takeda & Kainosho, Methods in Molecular Biology (2012) 831: 71-84
[2] Schwarz et al., Nature Protocols (2007) 2: 2945-57
[3] Kim & Choi, J. Biotechnol (2000) 48: 27-32