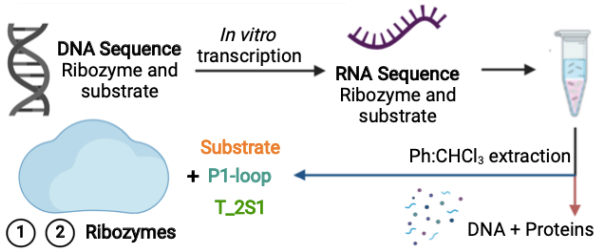
Materials and Methods
RNA World and the Origin of life

1. RNA Production



General scheme of RNA production for P1-Loop, T_2S1, substrate and ribozymes sequences

In vitro replication of main sequences of sunY and *Azoarcus* mutants were carried out in an Eppendorf Tube, using the following protocol in quantities and the later the PCR program used.

All sequences were preserved at -20°C before use.

	Stock solution	Unit	V(µl)	Final conc.	Unit
Ribozyme template	1	ng/µL	10	0,2	ng/µL
Buffer	5	X	10	1	X
Forward primer	10	µM	3,75	0,75	µM
Reverse primer	10	µM	3,75	0,75	µM
dNTP Mix	10	mM	1	0,2	mM
Polymerase (Phusion)	2	U/µL	0,5	0,02	U/µL
Water	-	-	21	50	
		Total	**50**		

PCR program:			
Step 1	98 °C	30 sec	
Step 2	98 °C	10 sec	**Denaturation**
Step 3	59 °C	30 sec	**Annealing**
Step 4	72 °C	30 sec	**Elongation**
Step 5	Go to step 2	30 times	
Step 6	72 °C	3 min	

After PCR, 2µL of each PCR product + 1µl stopping buffer were used for the agarose gel analysis. 25 mL of 2% gel were prepared by mixing 1 tablet of TopVision Agarose Tablets, containing 0,5 g agarose, and 25 mL TAE buffer 1X. Solution was heated in a microwave until it appeared clean. The gel was pre-run 10 minutes using the same buffer, at 12 V/cm gel was left 25 min. The purpose of analysis was to determine if the PCR cycles had been successful in replicating the sequence.

Only then RNA production can begin with the usage of HiScribe™ T7 High Yield RNA Synthesis Kit which uses the T7 RNA Polymerase. Protocol was followed as indicated by the fabricant each reaction was carried out in 20 µL. After the reaction purification was needed. It comprised several steps:

Phenol-chlorophorm extraction in acid medium:

Resuspension of the IVT solution with water (nuclease free 100µl) and transfer the solution into a 1,5 mL Eppendorf tube, under the hood 100 µL of phenol-chloroform pH 4 were add, vortex a few minutes, then centrifuge 4 minutes at 11 000 rpm, during the centrifugation, add 0.1 volume of 3M NaAc (sodium acetate) in new tubes at the end of the centrifugation, gently pipette the upper aqueous phase (containing RNAs) into the tubes containing NaAc add 2.5 volumes of cold 100% ethanol (stored at -20°C) and mix by up and down (the solution should become cloudy, RNAs precipitate quickly) let the RNAs precipitate at -20°C for 1h or overnight, centrifuge for 1h at 4°C 13 000 rpm, remove the supernatant, a white pellet should be visible. Wash the pellet with 150 µL of cold 70% ethanol (stored at -20°C) very gently (without dissolving the pellet) then centrifuge briefly and remove the supernatant. Repeat the wash a second time removing as much ethanol as possible cover the tube with parafilm (to prevent the pellet from escaping) and pierce some holes with a syringe dry the pellet in the concentrator under vacuum for a few minutes resuspend the pellet with 40 µL of water (pipette well until the pellet is dissolved)

DNAse I Treatment

We begin with 5 µl of DNase I buffer (10X) and add 5 µL of enzyme and incubate 15 min at 37°C immediately add 50 µl of stopping buffer. The sample can be stored at -20°C or can be deposited directly on extraction gel RNA extraction on acrylamide (PAGE) gel. Run the 8% acrylamide (PAGE) gel and preheat the gel (24W, 15 minutes). Denature the samples by heat treatment in thermocycler (5 minutes at 75°C) migration is done until the bottom marker is at the bottom of the gel (about 3 hours, 24W). Remove the gel, cut the top right edge to make a marker, and place it between 2 plastic films, place the gel on a silicon plate, illuminate at 254 nm and mark the outline with a marker, cut the gel with a razor blade. Place the gel pieces in the sample tubes, crush with a 1mL cone the gel on the wall of the tube and add 0.5 mL of 0.3M NaAc. Incubation in Thermomix for 5 hours at 26°C, finally centrifugation at 450-500 rpm.

RNA purification by EtOH precipitation

Transfer the contents of the tubes (taking care not to over-pipette the gel ends) into COSTAR columns and centrifuge for 4 minutes at 11 000 rpm (-add 1µl of glycogen if necessary). Add 2.5 volumes of 100% EtOH cooled and incubate at -20°C for 1 hour. Centrifuge at 13 000 rpm at 4°C for 1 hour. Wash the pellets with 150µL of cold 70% EtOH. Evaporate the ethanol (under vacuum or hood), resuspend in 20µl of water and measure the concentration with the nanodrop.

2. Catalytic Assays

All catalytic assays were performed in the same fashion manner. According to the variability of experimental conditions several catalytic assays could be run with the same controls. All sequences were introduced leaving at the end the ribozyme when the start point was taken immediately. All Eppendorf tubes were kept in a thermostat at 40 °C for minutes. Only for the actual assay samples (2 µL) were taken at 0, 2, 4, 7 and 24 hours for SunY and 0, 0,5, 1, 2, 4, 7 and 24 hours for Azoarcus. For controls only initial and final points were acquired. These samples were left in 6 µL stopping buffer (10 mM EDTA, 80% formamide, 5% bromephenol blue, 5% xylene cyanol). Before loading of the samples to the gel denaturation by temperature was done in thermocycler at 80°C for 5 minutes.

Samples were analyzed in 18% PAGE (Polyacrylamide gel with 50% as denaturing agent). 40 mL of Polyacrylamide solution were prepared using 198 µL 10% APC and 66 µL TEMED as polymerization agents. 4 µL of sample were introduced by lane. 4 µL ladders of 1, 0,5 and 0,25 mM were used of molecular weights of 21, 25, 36, 46 and 55 bp for all cases except in P1-Loop-Azo ligation where 25 bp standard was replace by a 26 bp standard. Gel was pre-run for 10 min in TBE buffer 1X, at 25 V/cm gel was left around an hour. Visualization of the gel was done using SYBR Gold system with a Blue Tray.

Protocol for P1 Loop ligation (*Azoarcus* vs SunY experiment)

Catalytic assay				
Reagent	**Stock sol.**	**Units**	**V(µl)**	**Final conc.**
Ribozyme	16	µM	0,3	0,5
P1 Loop	100	µM	1,5	15
S1_LL	100,0	µM	1,5	15
MgCl2	240	mM	2,5	60
Water	up to		4,2	
		Sum	10	

Ribozyme + P1 Loop Control				
Reagent	**Stock sol.**	**Units**	**V(µl)**	**Final conc.**
Ribozyme	16	µM	0,3	0,5
P1 Loop	100	µM	1,2	15
S1_LL	100,0	µM	0,0	0
MgCl2	240	mM	2,0	60
Water	up to		4,6	
		Sum	8	

P1 Loop + S1 Control				
Reagent	**Stock sol.**	**Units**	**V(µl)**	**Final conc.**
Ribozyme	16	µM	0,0	0
P1 Loop	100	µM	1,2	15
S1_LL	100,0	µM	1,2	15
MgCl2	240	mM	2,0	60
Water	up to		3,6	
		Sum	8	

Ribozyme + S1 Contol				
Reagent	**Stock sol.**	**Units**	**V(µl)**	**Final conc.**
Ribozyme	16	µM	0,3	0,5
P1 Loop	100	µM	0,0	0
S1_LL	100,0	µM	1,2	15
MgCl2	240	mM	2,0	60
Water	up to		4,6	
		Sum	8,0	

P1 Loop Control				
Reagent	**Stock sol.**	**Units**	**V(µl)**	**Final conc.**
Ribozyme	16	µM	0,0	0
P1 Loop	100	µM	1,2	15
S1_LL	100,0	µM	0,0	0
MgCl2	240	mM	2,0	60
Water	up to		4,8	
		Sum	8	

S1 Control				
Reagent	**Stock sol.**	**Units**	**V(µl)**	**Final conc.**
Ribozyme	16	µM	0,0	0
P1 Loop	100	µM	0,0	0
S1_LL	100,0	µM	1,2	15
MgCl2	240	mM	2,0	60
Water	up to		4,8	
		Sum	8	

Ribozyme Control				
Reagent	**Stock sol.**	**Units**	**V(µl)**	**Final conc.**
Ribozyme	16	µM	0,3	0,5
P1 Loop	100	µM	0,0	0
S1_LL	100,0	µM	0,0	0
MgCl2	240	mM	2,0	60
Water	up to		5,8	
		Sum	8	

P1-Loop and T_2S1 analysis with A and B *Azoarcus* modifications were done in similar manner 10 µL for catalytic assays and 8 µL for controls. Only concentrations were modified. Assay **A-B-P1-Loop**: Substrates 15 µM. Assay **A-B-P1-Loop**: Substrate S1 15 µM + 2.5 µM T_2S1.

Protocol for SunY Mg optimization in Water

MgCl2 50 mM				
Reagent	**Stock sol.**	**Units**	**V(µl)**	**Final conc.**
SunY	16	µM	0,3	0,5
T_2S_RNA	100	µM	0,5	5
S1_LL	100,0	µM	1,5	15
MgCl2	240	mM	2,1	50
Water	up to		5,6	
		Sum	10	

MgCl2 75 mM				
Reagent	**Stock sol.**	**Units**	**V(µl)**	**Final conc.**
SunY	16	µM	0,3	0,5
T_2S_RNA	100	µM	0,5	5
S1_LL	100,0	µM	1,5	15
MgCl2	240	mM	3,1	75
Water	up to		4,6	
		Sum	10,0	

MgCl2 100 mM				
Reagent	**Stock sol.**	**Units**	**V(µl)**	**Final conc.**
SunY	16	µM	0,3	0,5
T_2S_RNA	100	µM	0,5	5
S1_LL	100,0	µM	1,5	15
MgCl2	240	mM	4,2	100
Water	up to		3,5	
		Sum	10	

Sun Y + T_2S Control				
Reagent	**Stock sol.**	**Units**	**V(µl)**	**Final conc.**
SunY	16	µM	0,3	0,5
T_2S_RNA	100	µM	0,4	5
S1_LL	100,0	µM	0,0	0
MgCl2	240	mM	3,3	100
Water	up to		4,0	
		Sum	8	

T_2S + S1 Control				
Reagent	**Stock sol.**	**Units**	**V(µl)**	**Final conc.**
SunY	16	µM	0,0	0
T_2S_RNA	100	µM	0,4	5
S1_LL	100,0	µM	1,2	15
MgCl2	240	mM	3,3	100
Water	up to		3,1	
		Sum	8	

Sun Y + S1 Contol				
Reagent	**Stock sol.**	**Units**	**V(µl)**	**Final conc.**
SunY	16	µM	0,3	0,5
T_2S_RNA	100	µM	0,0	0
S1_LL	100,0	µM	1,2	15
MgCl2	240	mM	3,3	100
Water	up to		3,2	
		Sum	8,0	

T_2S Control				
Reagent	**Stock sol.**	**Units**	**V(µl)**	**Final conc.**
SunY	16	µM	0,0	0
T_2S_RNA	100	µM	0,4	5
S1_LL	100,0	µM	0,0	0
MgCl2	240	mM	3,3	100
Water	up to		4,3	
		Sum	8	

S1 Control				
Reagent	**Stock sol.**	**Units**	**V(µl)**	**Final conc.**
SunY	16	µM	0,0	0
T_2S_RNA	100	µM	0,0	0
S1_LL	100,0	µM	1,2	15
MgCl2	240	mM	3,3	100
Water	up to		3,5	
		Sum	8	

SunY Control				
Reagent	**Stock sol.**	**Units**	**V(µl)**	**Final conc.**
SunY	16	µM	0,3	0,5
T_2S_RNA	100	µM	0,0	0
S1_LL	100,0	µM	0,0	0
MgCl2	240	mM	3,3	100
Water	up to		4,4	
		Sum	8	

Protocol for SunY Mg optimization in EPPS 30 mM

Volume	**10 µL**		**MgCl2**	**0 mM**	
name	**stock**	**unit**	**vol(µl)**	**final conc.**	**unit**
SunY	9	µM	0,6	0,5	µM
T-2S	100	µM	0,5	5	µM
S1_LL	100,0	µM	1,5	15	µM
MgCl2	240	mM	0,0	0	mM
Buffer	200	mM	1,5	30	mM
Water	up to		5,9		
		Sum	10,0		

Volume	**10 µL**		**MgCl2**	**25 mM**	
name	**stock**	**unit**	**vol(µl)**	**final conc.**	**unit**
SunY	9	µM	0,6	0,5	µM
T-2S	100	µM	0,5	5	µM
S1_LL	100,0	µM	1,5	15	µM
MgCl2	240	mM	1,0	25	mM
Buffer	200	mM	1,5	30	mM
Water	up to		4,9		
		Sum	10,0		

Volume	**10 µL**		**MgCl2**	**50 mM**	
name	**stock**	**unit**	**vol(µl)**	**final conc.**	**unit**
SunY	9	µM	0,6	0,5	µM
T-2S	100	µM	0,5	5	µM
S1_LL	100,0	µM	1,5	15	µM
MgCl2	240	mM	2,1	50	mM
Buffer	200	mM	1,5	30	mM
Water	up to		3,9		
		Sum	10,0		

Volume	**10 µL**		**MgCl2**	**75 mM**	
name	**stock**	**unit**	**vol(µl)**	**final conc.**	**unit**
SunY	9	µM	0,6	0,5	µM
T-2S	100	µM	0,5	5	µM
S1_LL	100,0	µM	1,5	15	µM
MgCl2	240	mM	3,1	75	mM
Buffer	200	mM	1,5	30	mM
Water	up to		2,8		
		Sum	10,0		

Volume	**10 µL**		**MgCl2**	**100 mM**	
name	**stock**	**unit**	**vol(µl)**	**final conc.**	**unit**
SunY	9	µM	0,6	0,5	µM
T-2S	100	µM	0,5	5	µM
S1_LL	100,0	µM	1,5	15	µM
MgCl2	240	mM	4,2	100	mM
Buffer	200	mM	1,5	30	mM
Water	up to		1,8		
		Sum	10,0		

CONTROLS

Volume	8 µL		MgCl2	100 mM	
name	stock	unit	vol(µl)	final conc.	unit
SunY	9	µM	0,4	0,5	µM
T-2S	100	µM	0,4	5	µM
S1_LL	100,0	µM	0,0	0	µM
MgCl2	240	mM	3,3	100	mM
Buffer	200	mM	1,5	30	mM
Water	up to		2,3		
		Sum	8,0	Sun Y + P1	

Volume	8 µL		MgCl2	100 mM	
name	stock	unit	vol(µl)	final conc.	unit
SunY	9	µM	0,4	0,5	µM
T-2S	100	µM	0,0	0	µM
S1_LL	100,0	µM	1,2	15	µM
MgCl2	240	mM	3,3	100	mM
Buffer	200	mM	1,5	30	mM
Water	up to		1,5		
		Sum	8,0	Sun Y + S1	

Volume	8 µL		MgCl2	100 mM	
name	stock	unit	vol(µl)	final conc.	unit
SunY	9	µM	0,0	0	µM
T-2S	100	µM	0,4	5	µM
S1_LL	100,0	µM	1,2	15	µM
MgCl2	240	mM	3,3	100	mM
Buffer	200	mM	1,5	30	mM
Water	up to		1,6		
		Sum	8,0	S1 + P1	

Volume	8 µL		MgCl2	100 mM	
name	stock	unit	vol(µl)	final conc.	unit
SunY	9	µM	0,4	0,5	µM
T-2S	100	µM	0,0	0	µM
S1_LL	100,0	µM	0,0	0	µM
MgCl2	240	mM	3,3	100	mM
Buffer	200	mM	1,5	30	mM
Water	up to		2,7		
		Sum	8,0	Sun Y	

Volume	8 µL		MgCl2	100 mM	
name	stock	unit	vol(µl)	final conc.	unit
SunY	9	µM	0,0	0	µM
T-2S	100	µM	0,4	5	µM
S1_LL	100,0	µM	0,0	0	µM
MgCl2	240	mM	3,3	100	mM
Buffer	200	mM	1,5	30	mM
Water	up to		2,8		
		Sum	8,0	P1	

Volume	8 µL		MgCl2	100 mM	
name	stock	unit	vol(µl)	final conc.	unit
SunY	9	µM	0,0	0	µM
T-2S	100	µM	0,0	0	µM
S1_LL	100,0	µM	1,2	15	µM
MgCl2	240	mM	3,3	100	mM
Buffer	200	mM	1,5	30	mM
Water	up to		2,0		
		Sum	8,0	S1	

Protocol for SunY Mg optimization in Tris-HCl 30 mM

Volume	10 µL		MgCl2	0 mM	
name	stock	unit	vol(µl)	final conc.	unit
SunY	9	µM	0,6	0,5	µM
T-2S	100	µM	0,5	5	µM
S1_LL	100,0	µM	1,5	15	µM
MgCl2	240	mM	0,0	0	mM
Buffer	200	mM	1,5	30	mM
Water	up to		5,9		
		Sum	10,0		

Volume	10 µL		MgCl2	25 mM	
name	stock	unit	vol(µl)	final conc.	unit
SunY	9	µM	0,6	0,5	µM
T-2S	100	µM	0,5	5	µM
S1_LL	100,0	µM	1,5	15	µM
MgCl2	240	mM	1,0	25	mM
Buffer	200	mM	1,5	30	mM
Water	up to		4,9		
		Sum	10,0		

Volume	10 µL		MgCl2	50 mM	
name	stock	unit	vol(µl)	final conc.	unit
SunY	9	µM	0,6	0,5	µM
T-2S	100	µM	0,5	5	µM
S1_LL	100,0	µM	1,5	15	µM
MgCl2	240	mM	2,1	50	mM
Buffer	200	mM	1,5	30	mM
Water	up to		3,9		
		Sum	10,0		

Volume	10 µL		MgCl2	75 mM	
name	stock	unit	vol(µl)	final conc.	unit
SunY	9	µM	0,6	0,5	µM
T-2S	100	µM	0,5	5	µM
S1_LL	100,0	µM	1,5	15	µM
MgCl2	240	mM	3,1	75	mM
Buffer	200	mM	1,5	30	mM
Water	up to		2,8		
		Sum	10,0		

Volume	10 µL		MgCl2	100 mM	
name	stock	unit	vol(µl)	final conc.	unit
SunY	9	µM	0,6	0,5	µM
T-2S	100	µM	0,5	5	µM
S1_LL	100,0	µM	1,5	15	µM
MgCl2	240	mM	4,2	100	mM
Buffer	200	mM	1,5	30	mM
Water	up to		1,8		
		Sum	10,0		

CONTROLS

Volume	8 µL		MgCl2	100 mM	
name	stock	unit	vol(µl)	final conc.	unit
SunY	9	µM	0,4	0,5	µM
T-2S	100	µM	0,4	5	µM
S1_LL	100,0	µM	0,0	0	µM
MgCl2	240	mM	3,3	100	mM
Buffer	200	mM	1,5	30	mM
Water	up to		2,3		
		Sum	8,0	Sun Y + P1	

Volume	8 µL		MgCl2	100 mM	
name	stock	unit	vol(µl)	final conc.	unit
SunY	9	µM	0,4	0,5	µM
T-2S	100	µM	0,0	0	µM
S1_LL	100,0	µM	1,2	15	µM
MgCl2	240	mM	3,3	100	mM
Buffer	200	mM	1,5	30	mM
Water	up to		1,5		
		Sum	8,0	Sun Y + S1	

Volume	8 µL		MgCl2	100 mM	
name	stock	unit	vol(µl)	final conc.	unit
SunY	9	µM	0,0	0	µM
T-2S	100	µM	0,4	5	µM
S1_LL	100,0	µM	1,2	15	µM
MgCl2	240	mM	3,3	100	mM
Buffer	200	mM	1,5	30	mM
Water	up to		1,6		
		Sum	8,0	S1 + P1	

Volume	8 µL		MgCl2	100 mM	
name	stock	unit	vol(µl)	final conc.	unit
SunY	9	µM	0,4	0,5	µM
T-2S	100	µM	0,0	0	µM
S1_LL	100,0	µM	0,0	0	µM
MgCl2	240	mM	3,3	100	mM
Buffer	200	mM	1,5	30	mM
Water	up to		2,7		
		Sum	8,0	Sun Y	

Volume	8 µL		MgCl2	100 mM	
name	stock	unit	vol(µl)	final conc.	unit
SunY	9	µM	0,0	0	µM
T-2S	100	µM	0,4	5	µM
S1_LL	100,0	µM	0,0	0	µM
MgCl2	240	mM	3,3	100	mM
Buffer	200	mM	1,5	30	mM
Water	up to		2,8		
		Sum	8,0	P1	

Volume	8 µL		MgCl2	100 mM	
name	stock	unit	vol(µl)	final conc.	unit
SunY	9	µM	0,0	0	µM
T-2S	100	µM	0,0	0	µM
S1_LL	100,0	µM	1,2	15	µM
MgCl2	240	mM	3,3	100	mM
Buffer	200	mM	1,5	30	mM
Water	up to		2,0		
		Sum	8,0	S1	

3. Image Processing

Processing was done in GelAnalyzer. Lanes were selected and use of the Ladder was used to determine the molecular weight of different fragments. Background estimation was done with rolling ball method and 5% pic fit. Deviation for error bars were done considering 10% of area outside of gaussian pic.