Validation and amplification of random DNA libraries with modified nucleobases for Click-SELEX

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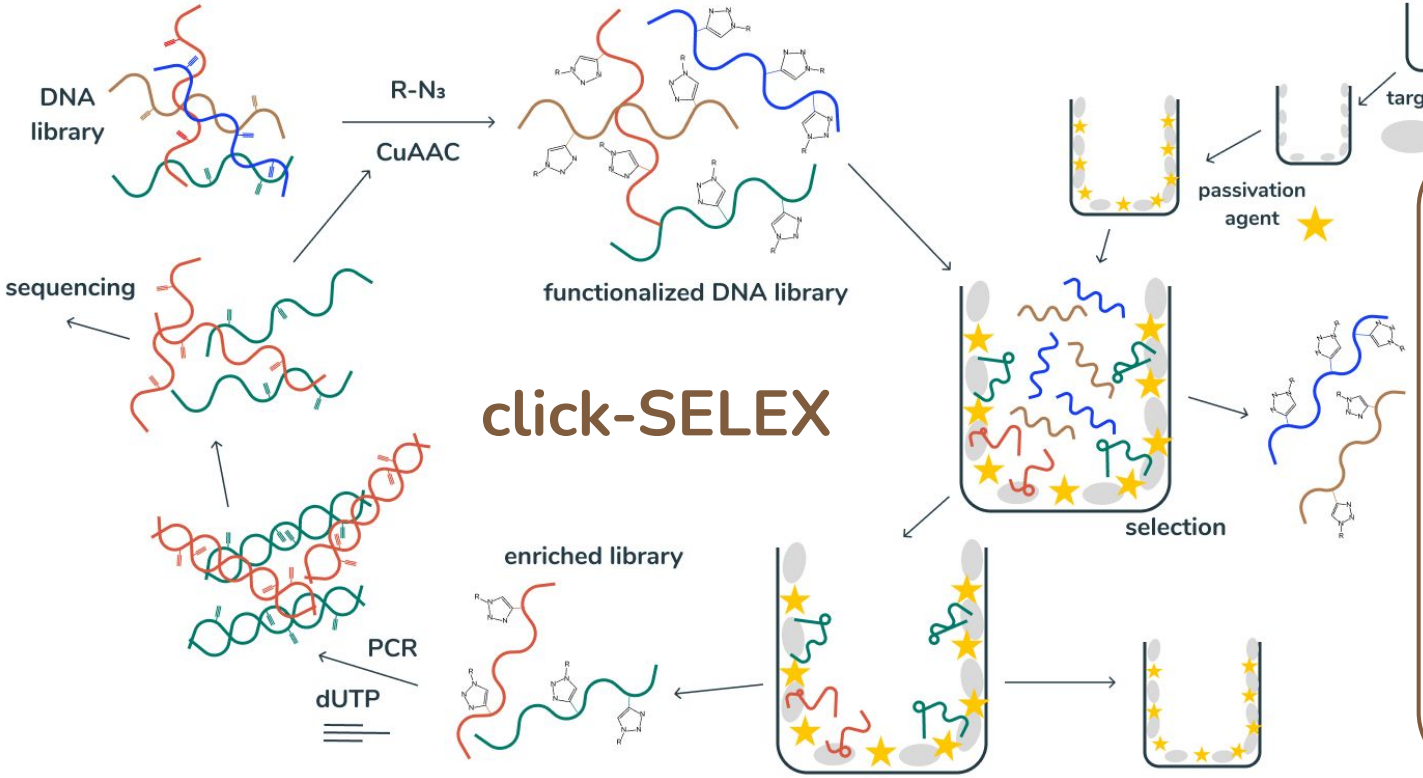

Features and benefits of click-SELEX:

- expansion of genetic alphabet;
- introduction of hydrophobic interactions the aptamer-target binding forces
- enlargement of target spectrum;
- increasing of binding affinity

Pfeiffer, F., Tolle, F., Rosenthal, M. *et al.* Identification and characterization of nucleobase-modified aptamers by click-SELEX. *Nat Protoc* 13, 1153–1180 (2018). https://doi.org/10.1038/nprot.2018.023

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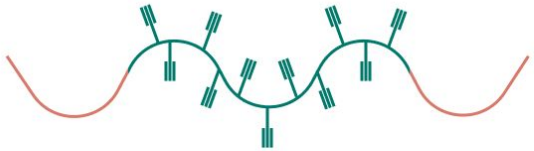
Objectives that were identified in the course of the research:

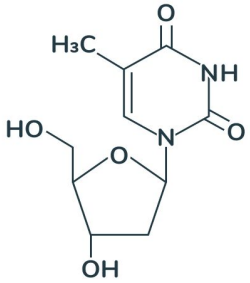
- Find out the relationship between the theoretical and actual ratio of nucleotides in the synthesis of the modified library
- Determine which azide can be quantitatively introduced into the DNA
- Identify the polymerase for PCR amplification with the modified DNA is a template
- Optimize PCR conditions

Library design: adjustment of nucleobase distribution

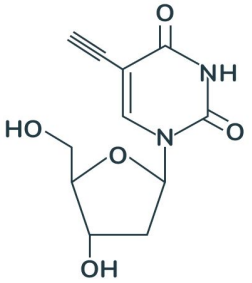


CL_40 5'-CACGACGCAAGGGACCACAGG-N40-CAGCACGACACCGCAGAGGCA-3'
CL40-For 5'-CACGACGCAAGGGACCACAGG-3'
CL40-Rev 5'-TGCCTCTGCGGTGTCGTGCTG-3'

Thymidine



5-Ethynyl-2'-deoxyuridine



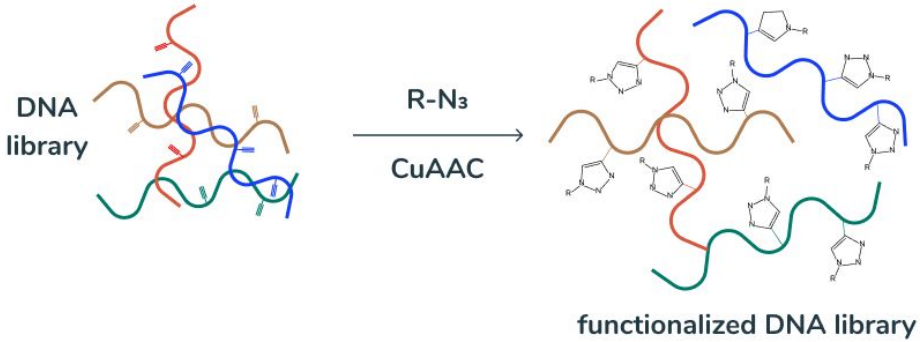
- Commercially available library consisted of oligonucleotides with a randomized central region and two fixed primer regions at both ends.
- Random region contained alkyne-modified uridine (5-ethynyl-deoxyuridine (EdU)) instead of thymidine.
- As nucleobases have different tendency to integrate into the DNA chain during synthesis, library was obtained in four variants with different distribution of nucleobases in the random region.
- Deep Vent (-exo) and Q5 hot start polymerases were used for the amplification of the libraries before next generation Illumina sequencing.

Sequencing results

Sample		%A	%C	%G	%EdU (T)
CL40_1 A:C:G:EdU = 1:1:1:1	theoretical	25	25	25	25
	DV	25,5	26,5	28,8	18,5
	Q5	25,3	26,0	29,3	18,6
CL40_2 A:C:G:EdU = 1.5:1.5:1.0:1.2	theoretical	28,8	28,8	19,2	23,1
	DV	28,9	21,1	32,8	16,4
	Q5	28,8	20,7	33,3	16,4
CL40_3 A:C:G:EdU = 1.5:1.25:1.15:1.0	theoretical	30,6	25,5	23,5	20,4
	DV	31,2	24,6	29,6	13,9
	Q5	31,2	24,2	30,1	13,9
CL40_4 A:C:G:EdU = 1.3:1.25:1.45:1	theoretical	26	25	29	20
	DV	26,2	29,6	29,5	14,0
	Q5	26,2	29,2	29,9	14,0

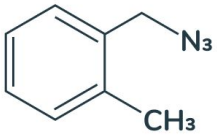
- NGS of alkyne-containing libraries confirmed the correct length, high diversity of the libraries and the uniformity of nucleotide distribution per position.
- No significant difference between the sequenced samples amplified with two different DNA polymerases (Q5 and Deep Vent (-exo)) was recognized.
- In all four libraries the content of EdU was lower than it was assumed during the chemical synthesis
- To reach the equimolar distribution of nucleobases, a higher proportion (more than 25%) of EdU should be used during the chemical synthesis of the library.
- Among the libraries studied, the distribution of bases towards equimolar is observed for the **CL40_1** library.

Library functionalization: azide screening

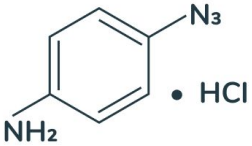


- This technology assumes adding of azide-bearing modifier of choice to alkyne-modified random DNA library using click-chemistry.
- Four substances were chosen for the purpose of studying.

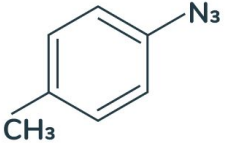
1-(Azidomethyl)-2-methylbenzene



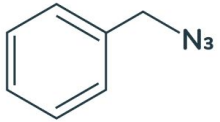
4-azidoaniline hydrochloride



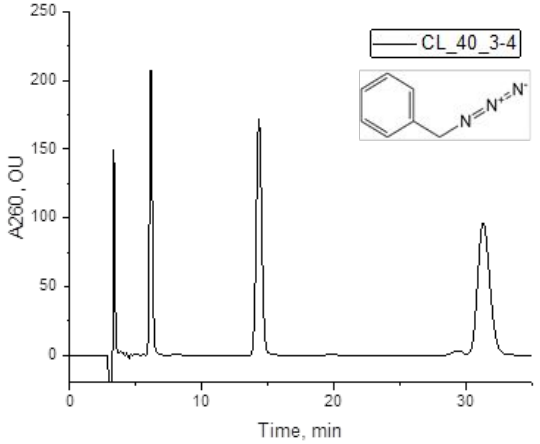
4-Azidotoluene

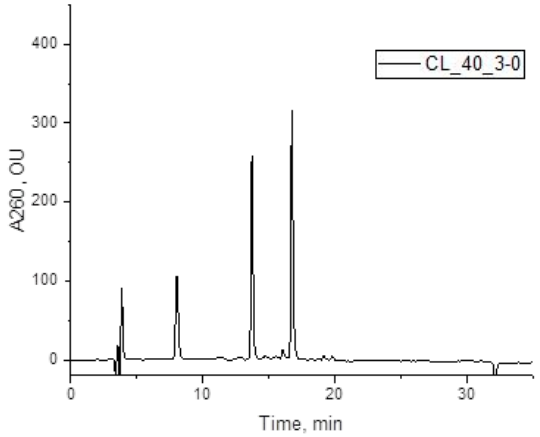


Benzyl azide



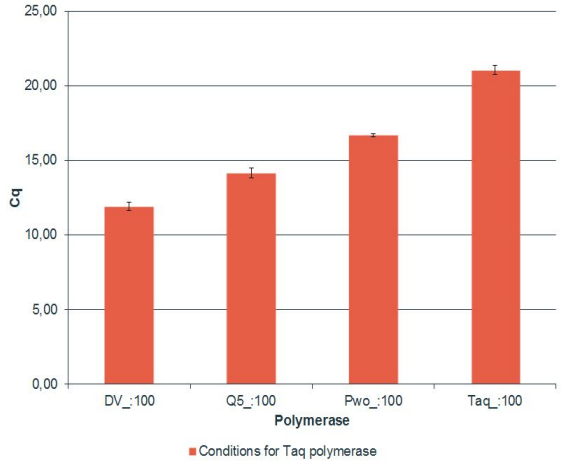
Azide introduction

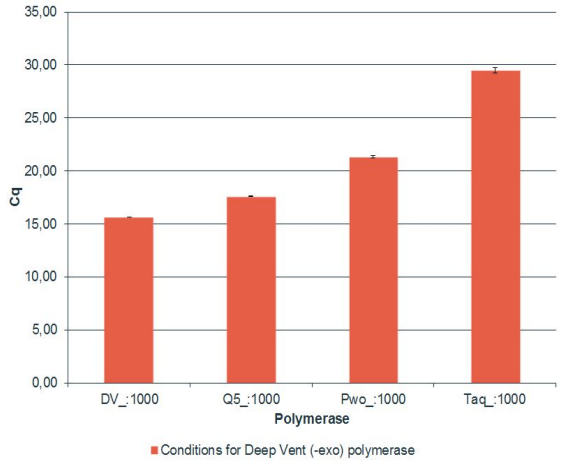




- Modified-DNA samples as well as the original library as a control, were digested to nucleosides using several enzymes (S1 nuclease, phosphodiesterase I, benzonase, alkaline phosphatase).
- RP-HPLC in gradient mode was used to separate the samples in 20 mM ammonium acetate solution with pH 4.5 and acetonitrile.
- Of the four modifiers we studied, only benzyl azide successfully functionalized the library.

Amplification of modified libraries





- The activity of various DNA polymerases (Q5, Deep Vent, Pwo, Taq) was screened for the amplification reaction of an alkyne-containing and functionalized library.
- During amplification followed by visualization on a native 8% PAGE gel, it was found that all polymerases were able to amplify an azide-containing DNA template, inserting both dNTP and EdUTP into the synthesized strand, and also use benzyl azide-modified DNA as a template. In all cases, the amplification efficiency decreased in the series Deep Vent > Pwo > Q5 > Taq.
- For a more accurate evaluation of amplification efficiency, qPCR was performed using various conditions according to the manufacturer's recommendations. The amplification efficiency decreased in the series Deep Vent > Q5 ≥ Pwo > Taq.

PCR optimization

Q5 Hot Start High Fidelity DNA polymerase		
Component	Volume (mkl)	Final concentration
Q5 Reaction Buffer	5	1x
dNTP Mix 10 mM	0,5	200 mkM
Upstream primer 10 mkM	1,25	0,5 mkM
Downstream primer 10 mkM	1,25	0,5 mkM
DNA template	1	
Q5 Hot Start High Fidelity DNA polymerase	0,25	0,02 U/mkl
5x Q5 High GC Enhanser	5	1x
Nuclease-free water	10,75	
	25	
98 (30'') - 98 (10") - 72 (15") 8 cycles		

Deep Vent (-exo) DNA polymerase		
Component	Volume (mkl)	Final concentration
ThermoPol Reaction Buffer 10x	5	1x
dNTP Mix 10 mM	0,5	200 mkM
Upstream primer 2 mkM	1,25	0,5 mkM
Downstream primer 2 mkM	1,25	0,5 mkM
DNA template	1	
Deep Vent (-exo) DNA polymerase	0,25	0,02 U/mkl
$MgSO_4$ 50 mM	5	1x
Nuclease-free water	10,75	
	25	
95 (30') - 95 (15") - 62 (30") - 72 (20'') 8 cycles		

Conclusions

- To reach the equimolar distribution of nucleobases, a higher proportion (more than 25%) of EdU should be used during the chemical synthesis of the library.
- Of the four modifiers we studied, only benzyl azide successfully functionalized the library. For this reason it is necessary to verify the quality of the functionalization when choosing a modifying azide for the study.
- All polymerases were able to amplify an azide-containing DNA template, inserting both dNTP and EdUTP into the synthesized strand, and also use benzyl azide-modified DNA as a template. Deep Vent (-exo) DNA polymerase demonstrated the best results.

Thank you for your attention!

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