In-vitro SELEX

Troubleshooting Guide

iGEM
Stockholm
2021

MIKROSKIN

INDEX

BEFORE THE EXPERIMENTS

In this section, we aim to guide future iGEM teams on how to order the essentials needed for SELEX, with special focus on the Library and primers ordering.

I. DESIGNING AND ORDERING A SELEX LIBRARY

SELEX libraries are collections with random sequences of DNA or RNA (aptamers). Each sequence should contain the random oligo sequence (40-60 nt), that constitutes the aptamer, but also overhangs on both the 5′ and 3′ end. I.e. this is our library sequence:

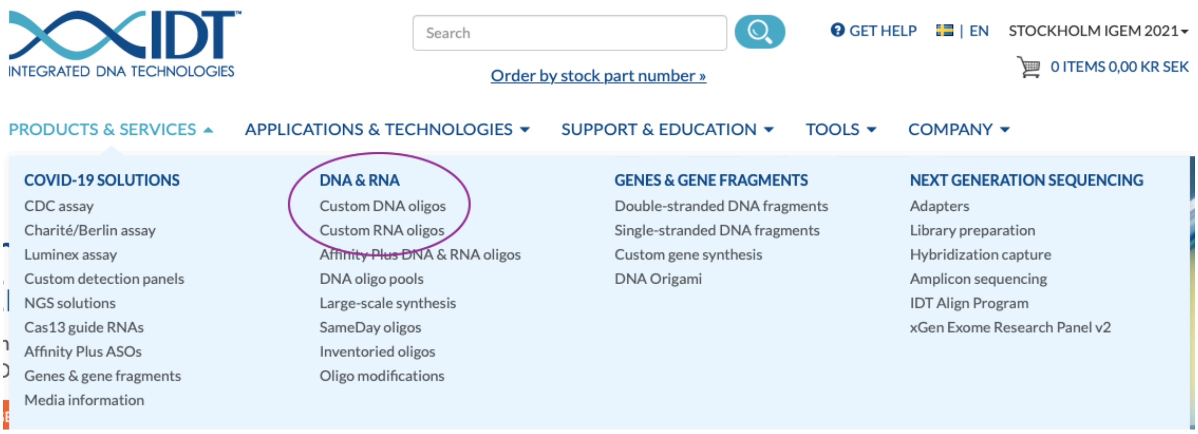
5′-ATCCAGAGTGACGCAGCA(N)(N)(N)(N)(N)(N)(N)(N)(N)(N)(N)(N)(N)(N)(N)(N)(N)(N)(N)(N)(N)(N)
(N)(N)(N)(N)(N)(N)(N)(N)(N)(N)(N)(N)(N)(N)(N)(N)(N)(N)TGGACACGGTGGCTTAGT-3′

5′ and 3′ overhangs should have a known sequence, since they will be used for primer design and later, for the PCR amplification of the aptamers that bind to the target of interest (library enrichment).

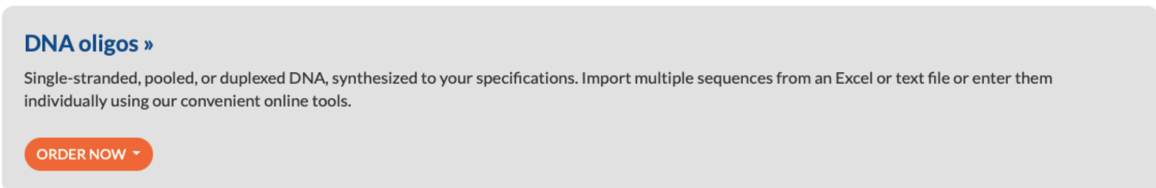
How to order a SELEX library on IDT - step by step

1. Enter on the IDT website (https://eu.idtdna.com/pages)

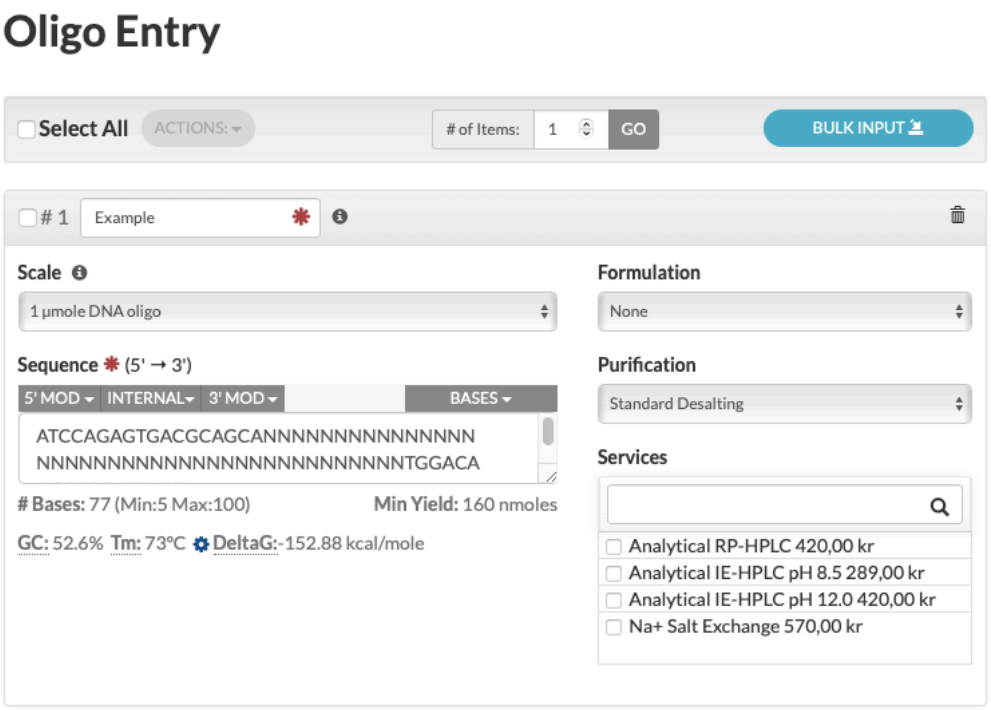
2. Click on Products and Services > Custom DNA/RNA oligos



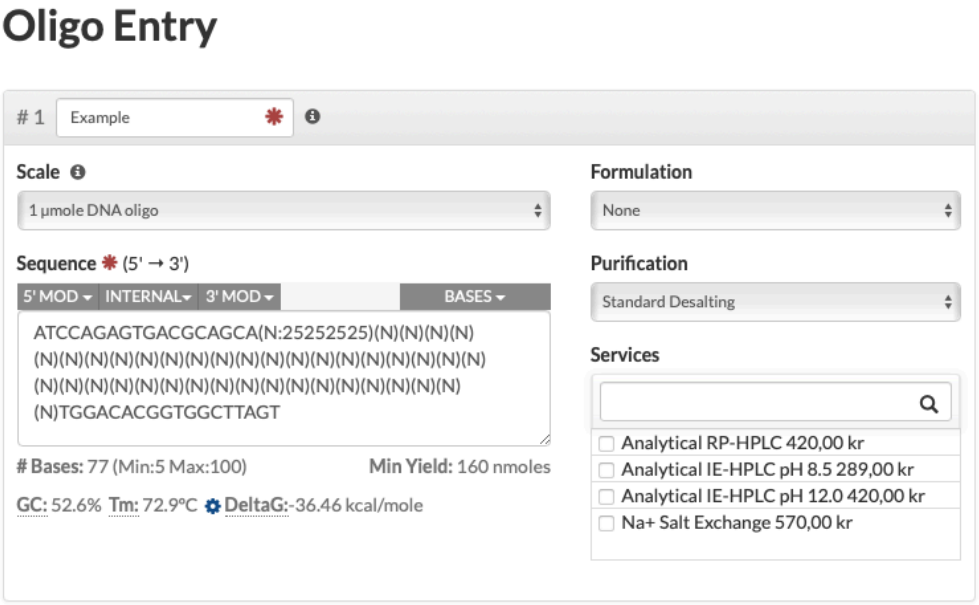
3. DNA oligos > Order now > Order in tubes



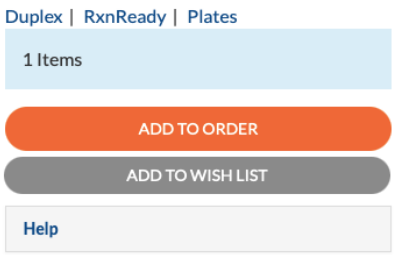
4. In Oligo Entry, write the name and sequence of your DNA oligo and the scale (mol) of the product.
   Purification > Standard Desalting



5. To get Unique Handmix Ratio in the N bases:
   1) Put every N between brackets → (N)
   2) In the first (N) in your sequence, write: (N:25252525)
      This way the Ns in your sequences will be filled using a mixed base pool with 25% of each base (A, T, C, G)

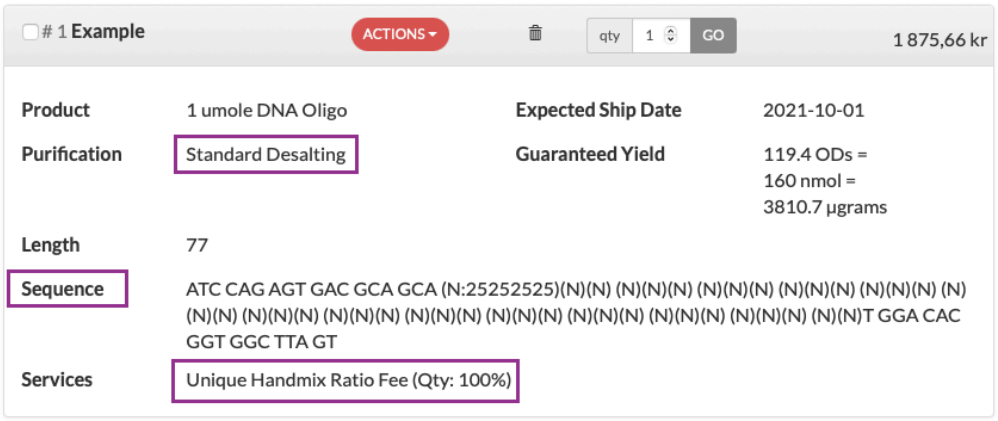


6. Add to order (right side of the screen)
   > Continue

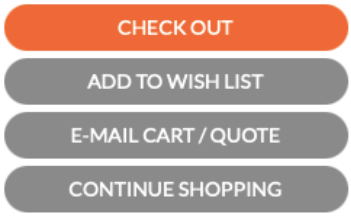


7. Double check:

   1) Purification > Standard Desalting
   2) Sequence: check if your sequence and sequence length is correct
   3) Services > Unique Handmix Ratio



8. Check out.
   Fill in your Shipping and Billing details and finalise the order.



II. DESIGNING AND ORDERING PRIMERS

Primers are used to amplify by PCR the aptamers that bound to the target during the selection round.

Both primers need to include modifications in their 5′ end in order to be able to differentiate between the sense and antisense DNA strand after the PCR and select the sense ssDNA strand.

Forward primer

- Same sequence as the 5′ overhang.
- 5′ Fluorescent dye modification, i.e. 6-FAM (Fluorescein) → will label the sense strand.

I.e. our Forward primer sequence is: /56-FAM/ATC CAG AGT GAC GCA GCA

Reverse primer

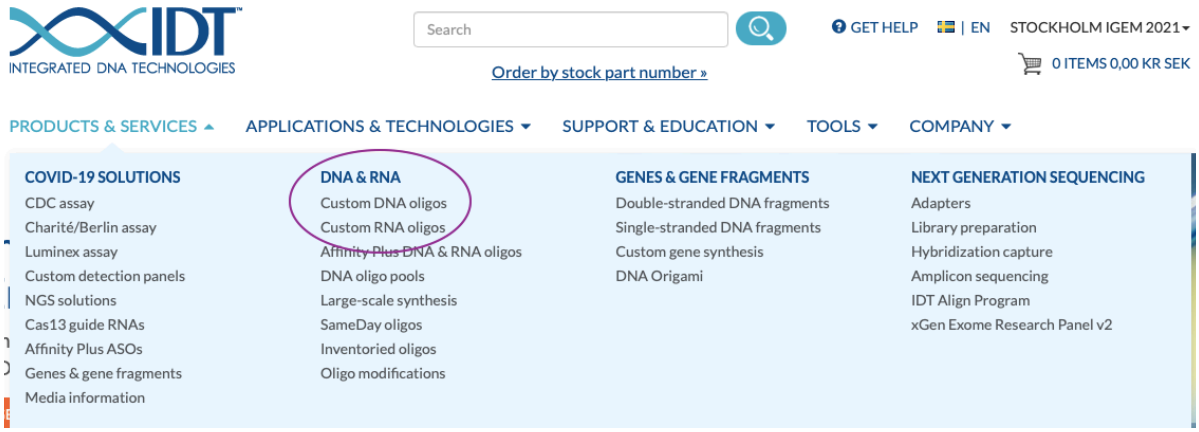
- Sequence is the reverse complementary sequence of the 3′ overhang
- 5′ Biotin modification → will label the antisense DNA strand.

I.e. our Reverse primer sequence is: /5BiosG/ACTAAGCCACCGTGTCCA

Taking advantage of the Streptavidin - Biotin affinity we can remove the antisense DNA strand from the library that will be used in the next SELEX round. I.e. by using Streptavidin beads.

How to order primers on IDT - step by step

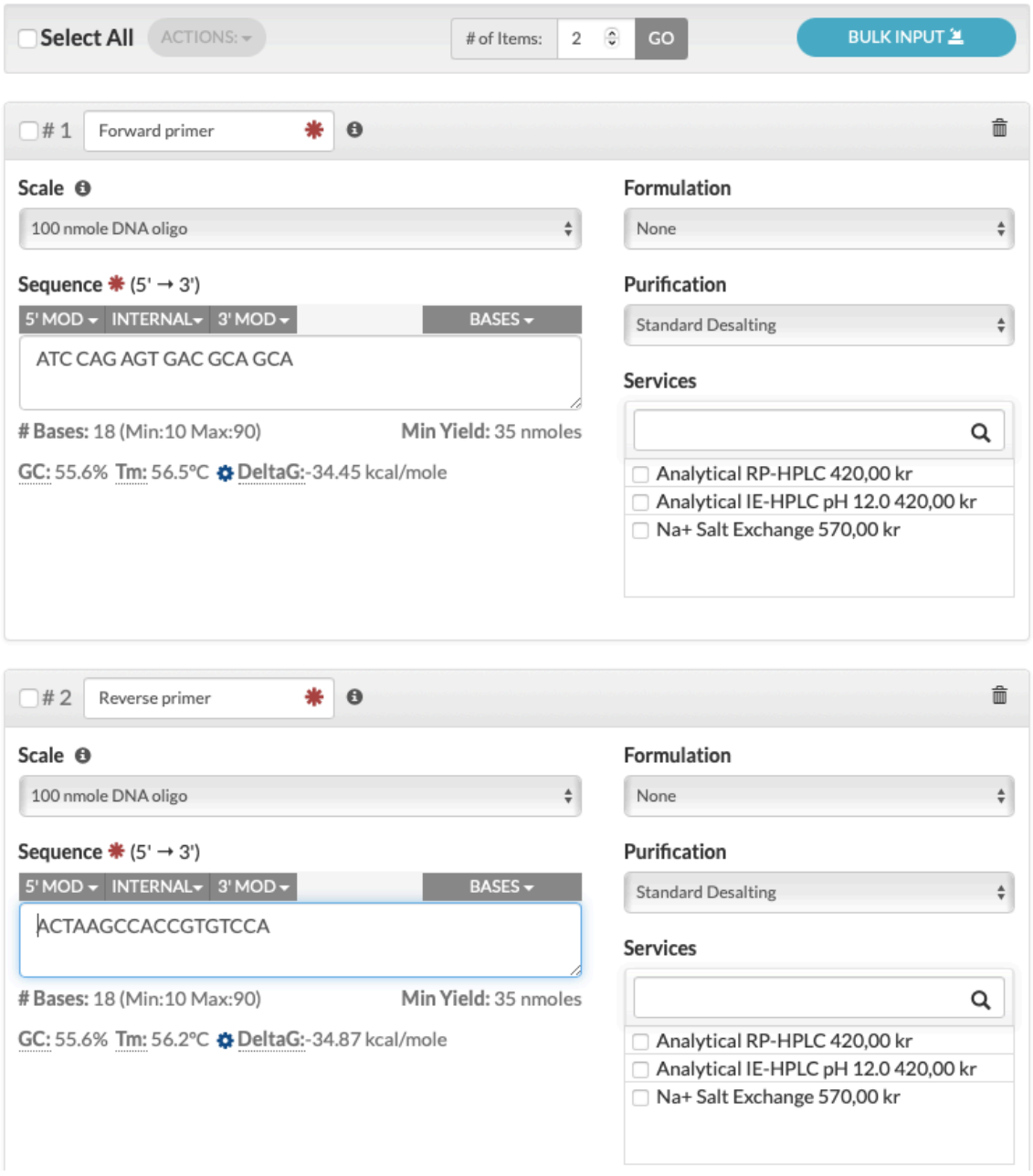
1. Enter on the IDT website (https://eu.idtdna.com/pages)
2. Click on Products and Services > Custom DNA/RNA oligos



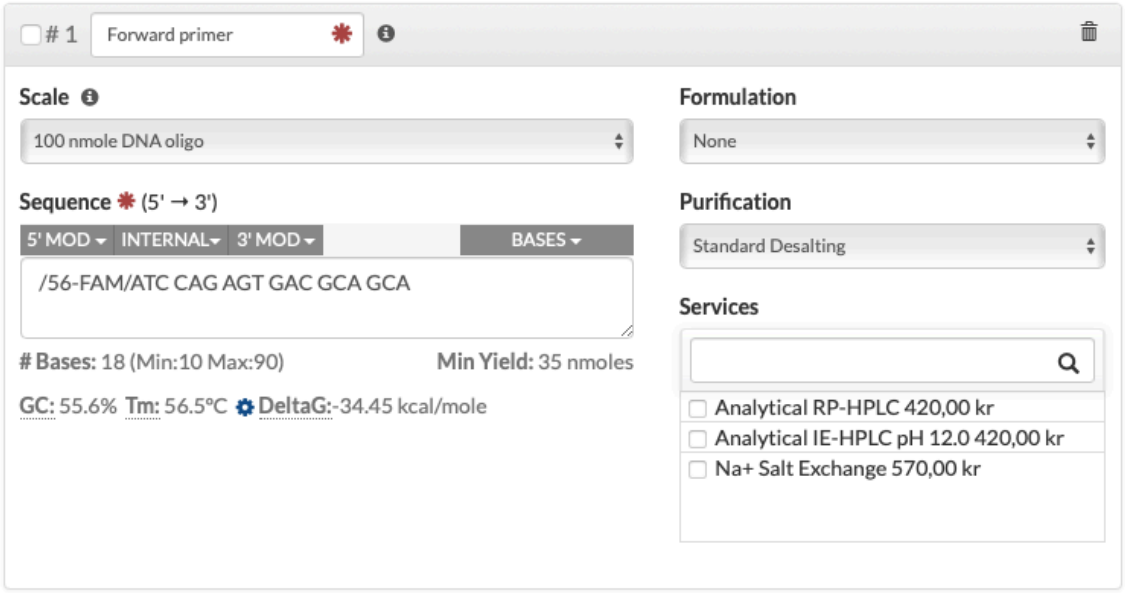
3. DNA oligos > Order now > Order in tubes
4. In Oligo Entry, increase the "# of items" to 2. Then, write the names and sequences of your primers and the scale (mol) of the product.

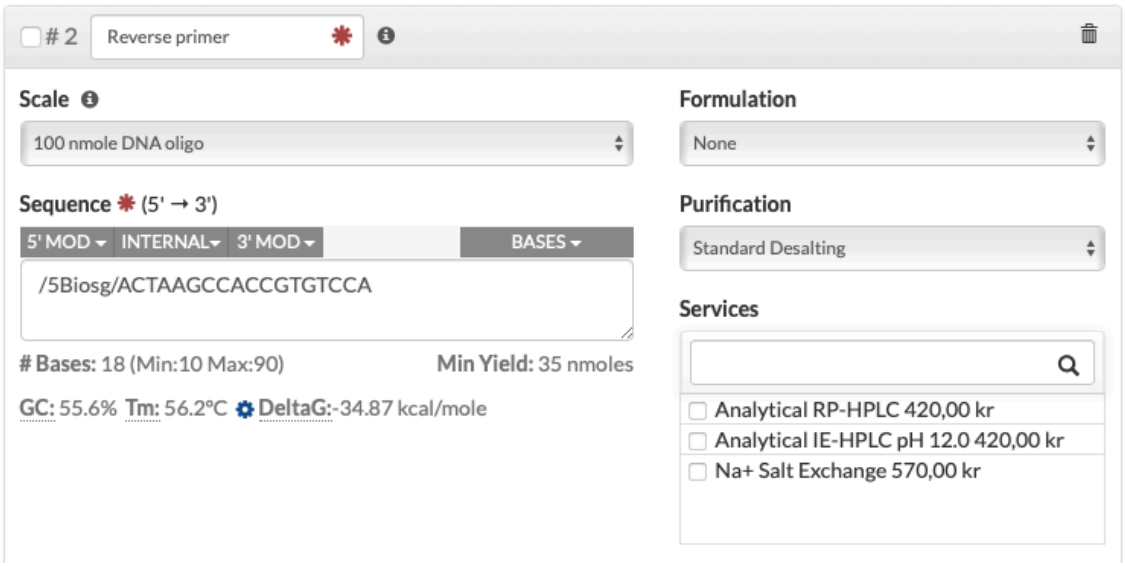
   ⚠️ *Make sure that you are writing the Reverse primer sequence correctly: reverse complementary sequence of the 3' overhang, but also from 5' to 3' end.*

   Purification > Standard Desalting

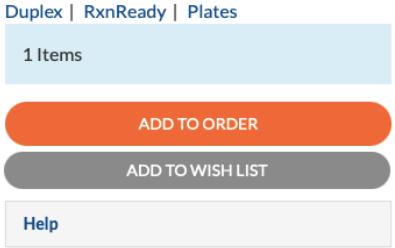


5. Add the 5’ modifications by clicking on “5’ MOD”
    - For the Forward primer, choose “/56-FAM/”
    - For the Reverse primer, choose “/5BiosG/”





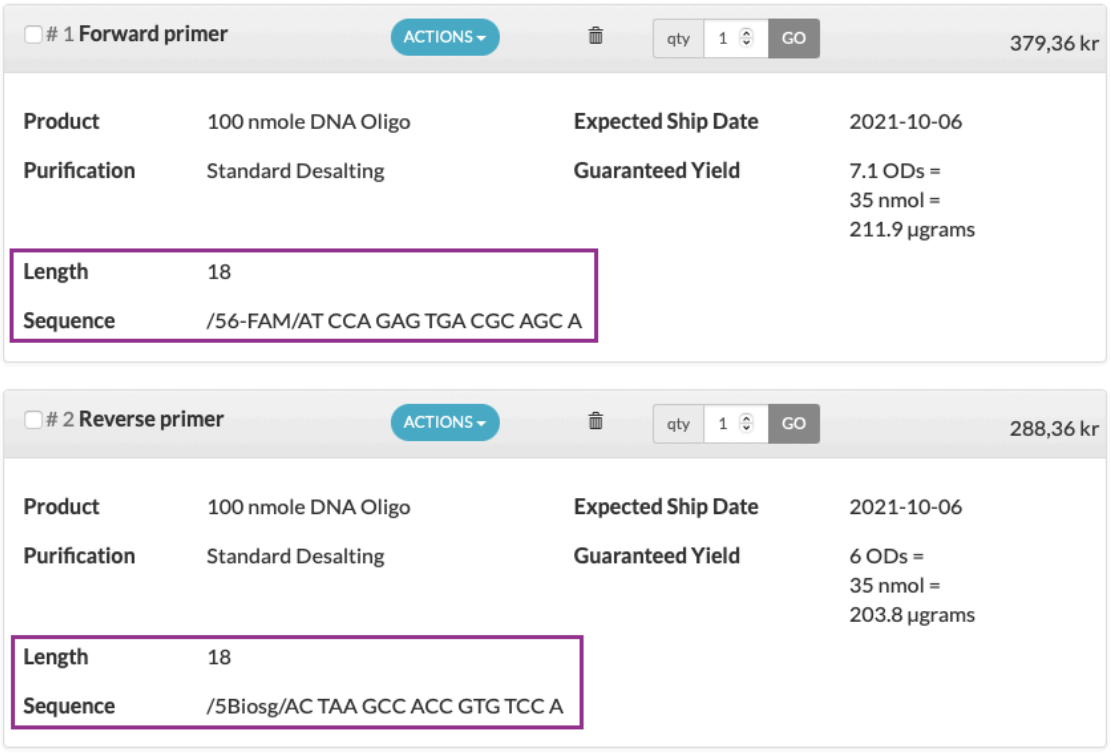
6. Add to order (right side of the screen)
   > Continue



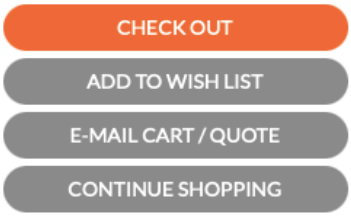
7. Double check:

    1) Purification > Standard Desalting

    2) Sequence: check if your sequence, modifications and sequence length is correct



8. Check out.
   Fill in your Shipping and Billing details and finalise the order.



III. OTHER NECESSARY PRODUCTS

Besides the library and the primers, other products should also be order before starting with the SELEX experiments:

- CML-Latex beads
  ↪ *to couple with your target of interest*
- Nuclease-free water

  ⚠️ *Nuclease-free water is used in multiple steps. In order to avoid contamination we recommend to prepare aliquots in 1.5 mL Eppendorf tubes and 15 mL Falcon tubes.*
- 24-well culture plates
  ↪ for Negative selection
- Sodium acetate (NaOAc)
  ↪ for DNA precipitation
- Ethanol 95%
  ↪ for DNA precipitation

  ⚠️ *Since it needs to be cold for the DNA precipitation, we recommend to store an aliquot in a 50mL Falcon at -20ºC.*
- Glycoblue
  ↪ for pellet visualisation after DNA precipitation
- Taq polymerase

  ⚠️ *In order to amplify and slightly modify the sequences in the library after the selection step in each SELEX round, a polymerase that lacks error correction (exonuclease 3'-5' activity) is needed.*
- dNTPs
  ↪ *for PCR*
- Agarose
  ↪ for DNA electrophoresis
- DNA gel stain (I.e. SybrSafe)
  ↪ for staining agarose gels in DNA electrophoresis
- Low range DNA ladder
  ↪ for DNA electrophoresis

- Streptavidin beads (i.e. Dynabeads)
  ↪ for separating the 5′ 6-FAM sense strand from the 5′ Biotin antisense strand after the PCR amplification
- Magnet rack
  ↪ for separation of sense from antisense ssDNA bound to Dynabeads
- Sodium hydroxide (NaOH)
  ↪ for eluting the sense ssDNA from the antisense ssDNA bound to the Dynabeads
- GE healthcare Cytiva illustra NAP column NAP-5
  ↪ for desalting the library after eluting the sense ssDNA from the antisense ssDNA (bound to the Dynabeads) with NaOH
- MES free acid
  ↪ for MES buffer
- Tris-base
  ↪ for TBE buffer
- Boric acid
  ↪ for TBE buffer
- EDTA
  ↪ for TBE buffer
- PBS
  ↪ for Buffer, solutions and washes
- BSA
  ↪ for Wash Buffer and coating plates (Negative selection step)
- Earle's Balanced Salt Solution
  ↪ for coating plates (Negative selection step)
- $MgCl_2$
  ↪ for Wash Buffer
- Yeast tRNA
  ↪ for Binding Buffer

⚠️ *Yeast tRNA can be purchased as a powder or liquid form. In case of acquiring the yeast tRNA powder, after resuspension, the solution should be frozen using liquid N_2 or dried ice and stored at -80ºC.*

IN-VITRO SELEX PROTOCOL

The SELEX protocol is very complex. Therefore, we went through every step and included a brief explanation of the rationale behind them as well as troubleshooting tips.

LIBRARY PREPARATION

✏️ *Set the heater to 95ºC in advance.*

✏️ *Calculate how much Binding Buffer you need (~ 2 mL) and prepare 0.5 mL extra, just in case.*

1. Let ssDNA pool denature by heating at 95°C for 5 min

   ⚠️ *This step allows DNA denaturation and breaks down secondary structures.*

2. Dissolve 20 pmol naive ssDNA library in 500 µl Binding buffer (BB)

   ▸ 20 µL of 1 µM library in 500 µL BB

   ⚠️ *Make sure you don't need a big volume of you library. Otherwise it will dilute the BB.*

3. Cool on ice for 10 min

   ⚠️ *This step allows the formation of stable tertiary structure.*

📍 *Stop point*

NEGATIVE SELECTION → Don't do in the first SELEX round

4. Wash BSA coated cell culture plate 2x 10 min with 500 µl Wash buffer (WB) by using a shaker

   ▸ See Related protocols > BSA coating of culture plates

5. Wash BSA coated cell culture plate 1x 10 min with 500 µl BB by using a shaker
6. Add 500 µl BB to cell culture plate
7. Add ssDNA library and incubate for 30 min on shaker at 4°C

   ▸ Supernatant contains unbound DNA sequences → New library ❗

POSITIVE SELECTION

✏️ *Prior to the positive selection, the target of interest should be conjugated to beads. Go through the literature and find the suitable protocol for the chemical properties of your target of interest. Also take into account the beads manufacturer's recommendations.*

8. Spin down 0.4 mg beads from stock solution at 1200g for 10 min
    - I.e. Stock 10 mg/ml → Take 40 µl and add 500 µl MES buffer
9. Wash beads 2x with 500 µl WB at 1200g for 10 min
10. Wash beads 1x with 500 µl BB at 1200g for 10 min
11. Resuspend beads in 500 µL BB
12. Mix dissolved library (500 µL) together with resuspended beads → 1mL in total
13. Incubate for 1h on rotator at 4°C
    - Place rotator in the fridge
14. Centrifuge at 1200g for 10 min
15. Wash 1x with 500µl WB at 1200 g for 10 min

📌 In later rounds: increase number of washing steps by one more wash and one more minute, until you reached 5 minutes of washing time.

ELUTION OF BOUND APTAMERS

✏️ *Set the heater to 95ºC in advance.*

16. Resuspend in 500µL BB
17. Heat at 95°C for 15min
18. Cool on ice for 5 min
19. Centrifuge at 14,000 rpm for 10 min → Supernatant contains aptamers

❄️*Freeze point*

DNA PRECIPITATION

✏️ *Remember to have cold 95% EtOH → -20ºC*

20. Add 0.1V x 3M Sodium acetate to the supernatant from step 19
    - 50 µL 3M Sodium acetate for 500 µL
21. Add 100µg/mL Glycoblue to solution
    - 5 µL Glycoblue for 500 µL
22. Add 2.5V 95 – 99% EtOH, cold (- 20 °C)
    - 1.375 mL EtOH for 500 µL

    a) Incubate 1h at -20ºC

    b) Incubate O/N at -80°C
23. Centrifuge at 10.000g for 30 min at 4°C
24. Remove supernatant
25. Wash in 50 µL 70% EtOH

    ⚠️ *The EtOH volume should be the just enough to cover the pellet.*
26. Take as much supernatant as possible and let dry

    ⚠️ *You shouldn't be able to smell EtOH anymore. Shouldn't look glossy. Don't let it dry too much either otherwise pellet resuspension becomes harder.*
27. Resuspend in 30 µL of nuclease free water
28. Measure DNA concentration with Nanodrop

❄️ *Freeze point*

APTAMER LIBRARY AMPLIFICATION

I. Library pre-amplification

29. Prepare 1 × 50 μL PCR tube:

Component	50µL reaction	Work concentration
Forward primer (10µM)	5 µL	1 µM / 50 pmol
Reverse primer (10µM)	5 µL	1 µM / 50 pmol
dNTP mix (2.5 mM each)	5 µL	0.25 mM each
DNA template (~100 ng/µL)	1 µL	100 ng
Taq DNA Polymerase	-	1.25 U
Polymerase Buffer	-	1X
H2O (nuclease-free)	Up to 50 µL	-

⚠️ *Always keep tubes on ice*

⚠️ *Add polymerase last*

⚠️ *Check your polymerase product sheet to calculate how much DNA pol and DNA pol buffer are needed.*

⚠️ *Use filtered tips and be careful during the pipetting to avoid contamination.*

30. PCR protocol for Library pre-amplification:

Initial denaturation → 95ºC 3'

Denaturation → 95ºC 30''

Annealing → -ºC 30'' × 6 Cycles

Elongation → 72ºC 30''

Final elongation → 72ºC 3'

⚠️ Annealing temperature → General rule: the annealing temperature should be 3–5°C lower than the lowest Tm of the primers.

⚠️ Elongation time → For Taq polymerase, elongation time is 1 min per 1 kb DNA, minimum elongation time being 30".

⚠️ Temperatures and times in the PCR protocol were selected based on our Taq polymerase. Always check the recommended protocol in the User manual from your manufacturer and adapt them accordingly.

❗ Now you have a new library!!!

❄️ *Freeze point*

II. Library amplification

31. Prepare 4 tubes × 50 μL PCR tube:
    - 3 tubes for the cycle testing → optimising the amount of cycles for your library is needed. Each tube will be run for different amount of cycles and the resulting amplifications will be compared.
    - 1 tube for NTC (Non Template Control) → 0 μL DNA template

Component	50µL reaction	Work concentration
Forward primer (10µM)	5 µL	1 µM / 50 pmol
Reverse primer (10µM)	5 µL	1 µM / 50 pmol
dNTP mix (2.5 mM each)	5 µL	0.25 mM each
DNA template (~100 ng/µL)	1 µL	100 ng
Taq DNA Polymerase	-	1.25 U
Polymerase Buffer	-	1X
H2O (nuclease-free)	Up to 50 µL	-

⚠️ *Always keep tubes on ice.*

⚠️ *Add polymerase last.*

⚠️ *Use filtered tips and be careful during the pipetting to avoid contamination.*

⚠️ *Preparing a Master mix also prevents contamination and is less time consuming.*

32. PCR for Library amplification:

Initial denaturation → 95ºC 3'

Denaturation → 95ºC 30''

Annealing → -ºC 30'' × 35 Cycles

Elongation → 72ºC 30''

Final elongation → 72ºC 3'

- Take out one tube after: 20, 25, 30 and 35 cycles

⚠️ Annealing temperature → General rule: 3–5°C lower than the lowest Tm of the primers.

⚠️ Elongation time → For Taq polymerase, elongation time is 1 min per 1 kb DNA, minimum elongation time being 30".

⚠️ Temperatures and times in the PCR protocol were selected based on our Taq polymerase. Always check the recommended protocol in the User manual from your manufacturer and adapt them accordingly.

❄️ *Freeze point*

33. Run 2 µL of each PCR product on a 2% agarose, 120V for 15 min

⚠️ See 2% agarose gel in TBE

- 2% agarose gel: 1 g agarose, 50 mL 1% TBE, 5 µL SybrSafe
- Low range DNA ladder: 2 µL Ladder (ready to use, dye not needed), 4 µL ddH_2O
- Samples: 2 µL sample, - µL DNA dye (working concentration 1X), - µL ddH_2O (up to 6 µL)

↪ Decide the **optimal PCR cycle** number based on the smearing in the gel. Choose number of cycles from the sample with less smeared and more clear band.

34. Repeat PCR library amplification (Protocol step 31, 32) with the number of cycles determined in step 33:

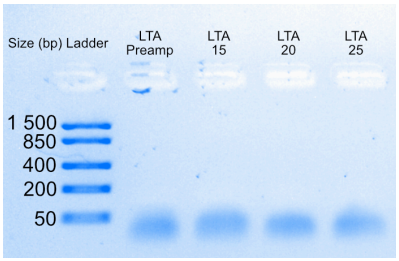
6 cycles (from pre-amplification) + *???* cycles (from amplification)

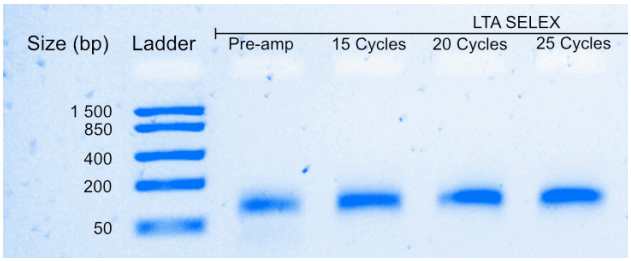
⚠️ *Do not prepare only one PCR tubes. Alternatively, prepare a Master Mix for several reactions in order to have a big amount of library, which is needed for the next step. It will also be helpful in case troubleshooting is needed and because.*

❄️*Freeze point*

📌 ❗ Based on our experience, optimising the PCRs in this step could take some time. Here we gathered some ***extra tips*** that could save you time along this process:

- We recommend spinning down the tubes before placing them in the thermocycler, to make sure that every drop is in the mix.
- Be extremely careful in order to avoid contamination of any of the PCR reagents. **If contamination appears**, prepare a new PCR tubes were you change one component at a time: i.e. tube 1 - new dNTPs, tube 2 - new Fwd primer, etc. Then run a gel and interpret the bands: the undesired band(s) will disappear in the sample where you replaced the contaminated component.
- **If there is no (or little) product in the gel and you can only see primer dimers**:





On the left image, all the bands correspond to primer dimers. On the right image, clear bands that correspond to the right size of our aptamer (~ 70 bp)

1) Check the final concentrations of every component. Your stocks might differ from ours, thus you cannot use the volumes we suggest in the tables. Look at the "Work concentration" column and adjust your volumes.
2) Check the annealing temperature. When primers are not annealing properly to the template DNA the PCR product will consist of primer dimers and little to no desired product. Check the annealing temperature of your primers in their respective IDT sheets. The annealing temperature should be 3–5°C lower than the lowest Tm of the primers. Using Tm calculator tools might be helpful, i.e. www.thermofisher.com/tmcalculator

- We recommend **optimising the PCR in pre-amplification step**. There is no need to waste reagents and time with following PCRs, you can continue once the PCR is working properly.
- If the optimisation is not being straightforward, **don't waste your eluted library** (previously incubated with the targets). Use the raw library instead and once the PCR is working move to the eluted library to continue with the SELEX cycle.
- **If you don't see clear bands** (there's still smear) when running "20, 25, 30 and 35 cycles" samples in the gel, check lower and higher number cycles. The optimal one might be before 20 or after 35!

SEPARATION OF SENSE AND ANTISENSE SSDNA

✏️ *In this step using the right proportion beads - DNA is key.*

✏️ *Measuring the DNA concentration with Nanodrop of a PCR product is not reliable. The sample contains PCR byproducts, primers, dNTPs, polymerase etc. In order to estimate the real DNA concentration in your sample you should compare the bands from the ladder and the PCR product in a gel image. Since the DNA concentration and loaded volume in the ladder are known, it is possible to make a rough DNA concentration estimation.*

3. Add the Streptavidin beads to the DNA

   ⚠️ *Prepare, wash and incubate the DNA with the beads according to the manufacturer.*

4. Add the magnets and discard the supernatant
5. Wash the beads + bound sequences with 500 μL WB
6. Elute the ssDNA from the beads by melting in a 0.1 M NaOH solution during 5'
7. Add the magnets and recover supernatant

   ⚠️ *Try to keep the volume under 600 μl. Keep in mind this will be your library for the next round and you don't want the aptamers to be very diluted.*

8. Desalt by using GE healthcare Cytiva illustra NAP column NAP-5
   - Run the protocol as given in the kit to filter out the NaOH
   - Recover the flow through

FLOW CYTOMETRY AND YIELD EVALUATION

9. Repeat binding step (see *Positive selection*)
10. Flow cytometry to assess library enrichment after the SELEX round

    ⚠️ *There is no need to perform flow cytometry in every SELEX round, especially at the beginning, since the increase in affinity will not be that big. It is recommended to do it every 2-3 rounds.*

📌 SELEX cycle 1 finished!
Repeat until the flow cytometry results show a plateau in the fluorescent intensity. Then, send the "plateau library" for sequencing to know your aptamers sequence. Probably, there will be different DNA species. Next step would be to perform a binding assay with each one of them separately to determine their Kd and choose the one with higher affinity.

RELATED PROTOCOLS

In this section protocols related to the SELEX cycle are included.

BSA COATING OF CULTURE PLATES

1. Prepare coating solution of BSA (10 µg/ml) in Earle's Balanced Salt Solution (sterile).
2. Pipette 100 μl of coating solution in a 24 well plate.
3. Swirl around to coat the base of the plate.

   ⚠️ *Make sure the coating solution covers the base of the plate evenly.*

4. Pipette the excess solution out.

   ⚠️ *You can use excess solution to coat for as many plates as needed) and again make sure the coating solution covers the bases of the plates.*

5. Cover the plate with the covers.
6. Incubate for 1-2 hours on a shaker.
7. Remove excess solution.
8. If not used immediately, store in PBS 1X at 4ºC.

2% AGAROSE GEL IN TBE

1. Prepare a 2% agarose solution in TBE in a beaker (or similar glass recipient).
2. Heat the beaker in the microwave until the solution boils.
3. Stir and heat again until reaching the boiling point again.
4. Stir and make sure all the agarose is dissolved and the solution is clear.
5. Add the DNA gel stain.
6. Stir until it is dissolved homogeneously.
7. Pour the solution into the cast (small 50 mL cast) and put the comb.
8. Remove bubbles with a tip and wait ~30 min for the gel to polymerise.

BUFFERS AND SOLUTIONS

We recommend to filter all buffers and solutions using a 0.2 μm filter to prevent contamination and maintain the samples' purity.

BUFFERS

Wash Buffer (WB)

- PBS (1X)
- BSA (1 mg/mL)
- 5mM $MgCl_2$

Binding Buffer (BB)

- yeast tRNA (0.1mg/mL)
  in WB

MES buffer (0.5M pH=6)

- MES free acid
- dH_2O

TBE buffer (pH=8.5)

- Tris-base 0.89 M
- Boric acid 0.89 M
- EDTA 25 mM
- ddH_2O

SOLUTIONS

Sodium acetate (3 M)

- NaOAc
- ddH_2O

Sodium hydroxide (0.1 M)

- NaOH
- ddH_2O