XELEX DNA SELEX CORE KIT

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PART 1: START OF SELECTION ROUNDS

KIT VERSION 1.0, MARCH 2013. SEE ALSO DETAILED VERSION OF THIS PROTOCOL.

A COUNTERSELECTION (OPTIONAL)

1. Counterselection (optional step)

→ Start with 1 - 3 µg purified dsDNA from the previous selection round.
(First SELEX round: 10 - 50 µg ssDNA library).
→ Mix dsDNA with 50 - 100 µl [10x] SELEX buffer (=1/10 final volume)
and fill up with nuclease-free water to 500 - 1000 µl final volume.
→ Denature dsDNA at 94°C for 3 min, then put immediately on ice.
→ Add to magnetic beads without immobilized target structure.
→ Incubate 1 h (round 1) or 15 min to 30 min (starting from round 2) at RT or 37°C.
→ Capture magnetic beads with a magnet.
Remove supernatant for use in the further selection process.

B APTAMER-TARGET BINDING

2. Binding

→ Immobilization: Bind target structure to beads (see separate protocol).
→ Start with 1 - 3 µg purified dsDNA from counterselection step
or from the previous selection round (First SELEX round: 10 - 50 µg ssDNA library).
→ Mix DNA (in water or elution buffer) with 50 - 100 µl [10x] SELEX buffer (=1/10 final volume)
and fill up with nuclease-free water to 500 - 1000 µl final volume.
→ Denature DNA at 94°C for 3 min, then put immediately on ice (Round 2 and later).
→ Add precooled DNA or ssDNA to magnetic beads with immobilized target structure
Amount of beads: Round 1: 10fold- (max. 200 µl), later rounds: 1fold binding capacity amount.
→ Add 500 µl 1x SELEX Buffer.
→ Incubate approx. 1 h (round 1) or 15- 30 min (round 2 and later).
Mix gently to prevent sedimentation of beads (gentle shaking or occasional pipetting).
→ Incubation temperature: Room temperature for analyses, 37°C for in-vivo targets

C APTAMER-TARGET WASHING

3. Washing

→ Round 1: 1 x with 1 ml SELEX Buffer
Round 2: 2 x with 500 µl – 1 ml SELEX Buffer
Additional rounds: Variable, roughly one additional washing step per additional selection round.
→ Varying and adjusting the selection stringency:
Adjust DNA / RNA and magnetic beads amounts, binding time,
number of washing steps, binding temperature and salt conditions.

D APTAMER-TARGET ELUTION

4. Elution (final volume 100 µl, choose one of the following alternate methods)

→ Direct PCR from DNA bound to beads
- Introduce beads directly into PCR reactions without elution. Applicable for 1 µM beads only.

→ Heat
- Heat beads in dest. H_2O + 2 mM EDTA to 70°C – 94°C for complete denaturation.
- Fix beads to one side of tube using a magnetic device, quickly (!) pipet the supernatant off.
- Transfer supernatant without any beads to a new reaction tube.
- Precipitate DNA or purify on DNA spin columns.

→ Heat + SDS (2 % [w/v])
- Incubate beads in 2 % [w/v] SDS and heat to 70°C – 94°C.
- Fix beads to one side of tube using a magnetic device, quickly (!) pipet the supernatant off.
- Transfer supernatant without any beads to a new reaction tube.
- Precipitate DNA or purify on DNA spin columns.

→ Elute DNA with Binding Buffer.
- Add 400 µl of orange-colored Orange-DX buffer and purify on DNA spin columns.

→ Competitive elution with either natural ligand of immobilized target for specific displacement or excess amount of free target.
The latter approach may fail to enrich high affinity binders.
→ For each selection round, keep backups of 50 % of (non-amplified) bead eluate.

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PART 2: EMULSION PCR PROTOCOL

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E EMULSION PCR – OIL PHASE PREPARATION

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5. Emulsion - Setting up the Oil Phase

→ Prepare 300 µl Oil Surfactant Mixture per reaction (50 µl water phase). Mix
220 µl Emulsion Component 1 (73 % [v/v])
20 µl Emulsion Component 2 (7 % [v/v])
60 µl Emulsion Component 3 (20 % [v/v])

→ Mix thoroughly by vortexing.

→ Keep on wet ice until further usage.

F EMULSION PCR – WATER PHASE PREPARATION

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6. Nucleic Acid Amplification of Binders (choose one of the following alternate methods)

→
- PCR / Taq DNA Polymerase
  (for DNA aptamer selection, protocol provided below)
- Mutagenesis PCR (see separate protocol)
  (for DNA aptamer selection, ships with separate protocol which replaces step 6a)
- NASBA / RNA Purification
  (for RNA aptamer selection, ships with separate protocol which replaces steps 6a - 14)

6a. Emulsion - Setting up the PCR Water Phase

→ SELEX PCR / Taq DNA Polymerase Protocol:
Water Phase: Mix 50 µl PCR sample on ice.
Template DNA: Introduce 50 - 130 pmol (= 1 – 3 µg) of purified dsDNA.

• 10 x PCR Buffer (with or without $MgCl_2$)	1 x	5 µl
• $MgCl_2$ [25 mM], if added separately	1.5 mM (or 1-5 mM)	3 µl (or 2- 10 µl)
• BSA, acetylated [10 mg/ml]	0.01 (or 0-1) mg/ml	0-5 µl
• Bank40-5'-Primer [100 µM]	4 µM (or 0.2 - 2 µM)	2 µl
• Bank40-3'-Primer [100 µM]	4 µM (or 0.2 - 2 µM)	2 µl
• dNTP mix [5 mM]	400 µM	4 µl
• Thermostable DNA Polymerase	2.5 U (or 1.25 - 2.5 U)	0.25 - 0.5 µl
• Template DNA (bead eluate, or use beads with bound DNA as template)	1 - 3 µg, up to 10^{13} copies	1-25 µl
• Sterile, DNA-free H_2O		@50 µl

G EMULSION PCR – MIXING OIL AND WATER PHASE

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7. Create Emulsion Reactions

→ Mix 300 µl Oil Surfactant Mixture (precooled, 4°C) and 50 µl Water Phase.

→ Vortex thoroughly for 5 minutes at 4°C on a vortexer with fixation aperture.

→ Dispense emulsion to three thin-walled reaction tubes ("triplicates") and run ePCR.

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8. Emulsion PCR - Parameters

→ Perform PCR reaction with the following parameters:
95°C - 120 sec - 15 - 20 x (95°C - 30 sec, 55°C - 60 sec, 72°C - 180 sec) - 72°C - 300 sec

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PART 3: END OF SELECTION ROUNDS

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H DNA PURIFICATION – BINDING STEP

9. Emulsion Breaking

- Apply 40 µl Activation Buffer SF to spin-column membrane, keep column at room temperature until usage, do not spin.
- Preheat Elution Buffer to 80°C (see section D, Elution).
- Pool triplicates of each sample into a 2 ml plastic reaction tube.
- Add 1 ml 2-butanol (or butanol) and break emulsion by vortexing.
- Add 500 µl of orange-colored buffer SFB (10 volumes of PCR water phase).
- Mix buffer completely with sample.
- Centrifuge for 2 min at 11 000 x g (approx. 12 000 rpm).

10. DNA Binding Step

- Remove organic phase (leave a small remain, do not remove interphase).
- Transfer water phase, interphase and remains of organic phase into a spin-column / receiver tube assembly.
- Centrifuge for 1 min at 11 000 x g (approx. 12 000 rpm).

I DNA PURIFICATION – WASHING STEPS

11. First Washing Step

- Discard flow-through and place back spin-column.
- Add 600 µl of Wash-SFX buffer to spin-column.
- Centrifuge for 1 min at 11 000 x g (approx. 12 000 rpm).

12. Second Washing Step

- Discard flow-through and place back spin-column.
- Add 350 µl of Wash-SFX buffer to spin-column.
- Centrifuge for 1 min at 11 000 x g (approx. 12 000 rpm).

13. Removal of Wash Buffer Traces

- Discard flow-through and place back spin-column.
- Centrifuge for 2 min at 11 000 x g (approx. 12 000 rpm) to remove any remaining traces of Wash-SFX buffer.

J DNA PURIFICATION – ELUTION STEP

14. DNA Elution

- Place spin-column in new collection tube (1.5 - 2 ml).
- Add 50-150 µl Elution buffer (optional: heated to 80°C).
- Incubate for 2 min at room temperature.
- Centrifuge for 1 min at 11 000 x g (approx. 12 000 rpm).
- Discard spin-column, cap the collection tube.
  Determine DNA concentration and total PCR yield per reaction.
- If total PCR yield is lower or much lower than 0.5 µg:
  Use the largest possible volume of purified DNA as template for scale-up emulsion PCR (20 PCR cycles, restart from step 5).
- If total PCR yield equals or is higher than 0.5 (better more than 1 µg):
  Use 10-50% of DNA for new selection round and keep 50-90% of DNA as backup.
- Go to step 1 or 2 to start new selection round (total 3 - 15 rounds) or end SELEX and start library / aptamer analysis (steps 14 - 17).

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PART 4: LIBRARY AND APTAMER ANALYSIS

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K DIVERSITY STANDARD PCR

15. Diversity Standard PCR amplification (non-emulsified PCR)

• 10 x PCR Buffer B (with 1.5 mM $MgCl_2$)	1 x	10 µl
• Bank40-5'-Primer [100 µM]	1 µM	1 µl
• Bank40-3'-Primer [100 µM]	1 µM	1 µl
• dNTP mix [5 mM]	400 µM	8 µl
• Thermostable DNA Polymerase [5U/µl]	2.5 U	0.5 µl
• Template DNA	0.5 µg, up to 10^{13} copies	
• Sterile, DNA-free H_2O	@100 µl	

16. Diversity Standard PCR - Parameters

→ Perform non-emulsified PCR reaction with the following parameters:
95°C - 120 sec - 2 - 4 x (95°C - 30 sec, 59°C - 30 sec, 72°C - 90 sec) - 72°C - 300 sec

L DiVE ASSAY

17a. DiVE Assay (alternate method to DiStRO assay)

→ Transfer 200 ng aliquots of standard or sample to separate plastic reaction tubes.
Duplicates: Four tubes per sample. Assay: S1 Nuclease treatment, control: untreated.

→ Add [5x] S1-Nuclease buffer to assay and control tubes (final concentration: 1x).
Mix thoroughly. Keep final volumes identical and as low as possible. Final volume : _____

→ Denature and reanneal both the assay and the control tubes:
- Denaturation: 3 min at 98°C
- Reannealing: 5 min at 65°C

→ Add 1 µl S1-Nuclease [1 U/µl] to the assay tube, but not to the control tube.
- Incubate for 30 min at 65°C.

→ Add 2 µl EDTA [0.5 M, pH 8.0] (final concentration: 2 mM).

→ Analyse 20-40 ng equivalent assay and control tubes side-by-side on 2-3 % agarose gels.

M DiStRO ASSAY

17b. DiStRO Assay (alternate method to DiVE assay)

→ Mix the following components:
300 ng of each diversity-standard or DNA-sample
+ 1 µl DA-buffer (10x)
+ 1 µl SYBR-Green (1:1000 in nuclease free H_2O)
Fill up to 10 µl with nuclease free H_2O

Real-Time-Cycler Program:
Denaturation: 2 min at 95°C
Annealing: 180 min at 76°C (or 2°C less than calculated Tm)
- Take one measurement per minute.

Remelting: Cool to 20°C, then heat by incrementing + 0,5°C per 7 sec to 98°C.
- Take one measurement per increment, i.e. per 7 sec.

N FLAA ASSAY (LIBRARY AND CLONE ANALYSIS)

18. FLAA Assay

→ Binder Assay: Bind biotinylated target to streptavidin-coated microtiterplate wells.
Add a 10-fold excess of target in relation to the denoted binding capacity.
Incubate 1 h at room temperature.
Negative control: Add 2 µl Biotin [5 mM]. Incubate for 10 min at room temperature.

→ Wash plate twice with 250 µl SELEX buffer [1x].

→ Dilute an appropriate amount of binder DNA in 50 µl SELEX Buffer [1x]. Amount : _____

→ Heat denature the DNA solution (95°C, 2 min) and immediately cool down on ice.
Transfer the precooled DNA solution to the target coated microtiter plate.
Incubate over night at 4°C or for one to two hours at room temperature.

→ Discard the DNA supernatant.
Wash plate immediately before measurement with 100 µl binding buffer [1x].

→ Add 50 µl Oligreen (or Picogreen) (1:500 in binding buffer) .
Measure twice after 9 min. (or after 4 min.): Ex. 485 nm, em. 527 nm.