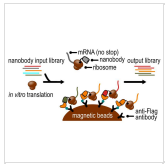

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CeVICA selection protocol

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ABSTRACT

The step-by-step protocol for performing the experimental steps of CeVICA, a cell-free nanobody selection platform. This protocol describes one round of selection. Multiple rounds of selection can be performed by iterating this protocol and using the output library DNA from the previous round as the input library DNA for the next round.

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KEYWORDS

CeVICA; nanobody; VHH; in vitro display; ribosome display; antibody

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MATERIALS TEXT

Buffer composition:
ISW buffer: 10mM HEPES, 150mM KCl, PH7.4 with 0.05% TritonX-100, 5mM $MgCl_2$, 0.1U/µl SUPERase.In, 1µg/µl BSA.

PBST: Phosphate-buffered saline with 0.02% Triton-X100

Primer sequences:
RT primer: CCAGTCGCTATAGCTGGCGTAGTTATATGAG
sRecovery pimer: AAACTAATACGACTCACTATAGGGTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAG
aRecovery primer: ATAGCTGGCGTAGTTATATGAGAAGATG

Reagents Ordering Information:
Protein G magnetic beads, ThermoFisher 10003D
Anti-Flag antibody, Sigmaaldrich F1804-50UG
BSA, NEB B9000S
*In vitro*transcription/translation kit, NEB E6800L
Monarch® RNA Cleanup Kit (10 µg), NEB T2030L
Maxima H- RTase, ThermoFisher EP0751
SUPERase.In,ThermoFisher AM2696
SPRIselect beads, Beckman Coulter B23317
Phusion DNA polymerase, NEB M0530L
Deep Vent DNA polymerase, NEB M0258L
NucleoSpin Gel and PCR Clean-Up kit, Takarabio 740609.250

1 Coat 100 µl Protein G magnetic beads with 4 µl anti-Flag antibody (diluted in 250 µl PBST) at room temperature for 1hour (see the manufacturer's protocol for more detail).

2 Wash antibody coated beads once with PBST. Then resuspend beads in PBST and aliquot equally into two tubes. One tube for pre-clearing, one tube for target immobilization.

3 Mix 100 µl cell lysate/media containing Flag tagged target protein with 150 µl PBST. Place the tube containing antibody coated beads on a magnet, discard PBST in the tube then resuspend beads in the lysate/media+PBST solution.

4 Rotate the tube at 4°C for 2 hours.

5 Briefly spin down tube in a mini-centrifuge, then wash beads three times with PBST by briefly pipetting. Then resuspend beads in 200 µl PBST. This is the target-bond beads.

6 *In vitro* transcription and translation (IVTL) reaction setup example, 25 µl IVTL with 250 ng CeVICA input library DNA, scale up as needed:

H2O	5 µl to 25 µl total
Solution A	10 µl
SUPERase.In	1 µl
CeVICA input library DNA	1.5 µl
Solution B	7.5 µl

7 Incubate the reaction in a 37°C incubator for 30 minutes.

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8 Place the IVTL reaction tube on ice, add 200 µl ice cold ISW buffer to the tube and mix by briefly pipetting. This is the stopped IVTL reaction (225 µl total).

9 Discard solution in the pre-clearing beads (antibody-coated beads), add stopped IVTL reaction to beads and resuspend beads.

10 Rotate the mixture at 4°C for 30 minutes.

11 Briefly spin down tube in a mini-centrifuge, then place the pre-clearing tube on a magnet.

12 Discard PBST in target-bond beads tube, transfer cleared IVTL solution from pre-clearing tube to target-bond beads and mix well.

13 Rotate the mixture at 4°C for 1 hour.

14 Wash beads with ISW buffer four times (briefly pipetting for the first wash, 5 to 10 minutes incubation at 4°C for the following three washes).

15 Briefly spin down the beads mixture tube in a mini-centrifuge then place the tube on a magnet. Discard solution then resuspend beads in 150 µl 1Xbinding buffer (100 µl binding buffer + 50 µl H2O, NEB Monarch RNA Cleanup Kit (10 µg)). Proceed to RNA extraction according to manufacturer's protocol and elute in 10 to 15 µl H2O.

16 Perform reverse transcription (RT) using Maxima H- RTase according to manufacter's recommendations: RT reaction setup example, scale up as needed:
H2O 3.42 µl to 10 µl total
5XRT buffer 2 µl
dNTP(10mM each) 0.5 µl
RT primer 10uM 0.25 µl
SUPERase.In 0.25 µl
Maxima H- RTase 0.25 µl
RNA 3.33 µl

17 RT reaction cleanup using SPRIselect beads: add 5mM Tris.HCl PH8.5 to each RT reaction to bring the total volume to 40 µl.

18 Place 26 µl (0.65X) SPRIselect beads in a tube and add the 40 µl RT solution to beads and re-suspend thoroughly by pipetting up and down 10 times, incubate at room temperature for 5 minutes.

19 Place the tube on a magnet for 1 minute and then discard liquid. wash pellet with the tube still on magnet by adding 180 µl 85% ethanol, incubate for 1 minute, then discard ethanol. Briefly spin the tube in a mini-centrifuge then put the tube back on a magnet and remove any residual ethanol with a 20 µl pipette tip.

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20 Dry beads by keeping the tube open for 1 to 5 minutes.

21 Elute by adding 17 µl water to beads and resuspend beads, incubate at room temp for 2 minutes then place the tube on a magnet for 1 minute and transfer 15 µl (Purified RT product) for PCR.

22 PCR amplification: PCR reaction setup example, scale up as needed, 3 X 50 µl PCR reaction:
H2O 51 µl
5XPhusion HF buffer 30 µl
100% DMSO 3 µl
Primers 45 µl (mixture containing 1uM each of sRecovery and aRecovery)
Phusion DNA polymerase 3 µl
Deep Vent DNA polymerase 3 µl
Purified RT product 15 µl
Thermal cycling conditions: 95°C 1 minute, (95°C 15 seconds, 60°C 15 seconds, 72°C 20 seconds)XN, 72°C 10 seconds. N is the number of cycles, which is typically 15-25, an appropriate N should be chosen such that sufficient PCR amplification product is obtained.

23 Purify PCR product using NucleoSpin Gel and PCR Clean-Up kit and elute in 12 to 20 µl Elution buffer. This is the output library DNA.

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