Harbor-IN NEON Electroporation SOP

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Purpose

This document outlines Demeetra's procedure for transfecting Harbor-IN transposase and transposon into CleanCut GS CHO cell lines using the Neon NxT electroporation system.

Required Materials

Product or Reagent	Vendor	Catalog No.
Fisherbrand™ Disposable PES Filter Units	Fisher Sci	FB12566506
GlutaMAX™ Supplement	Gibco	35050061
CD FortiCHO™ Medium	Gibco	A1148301
Anti-Clumping Agent	Gibco	0010057AE
Nunc™ Non-Treated 24-Well Plate	Thermo Sci	12-566-82
Nunc™ Non-Treated 6-Well Plate	Thermo Sci	12-566-80
25 cm^2 Rectangular Canted Neck Not Treated Cell Culture Flask (T-25)	Corning	431463
125 mL Polycarbonate Erlenmeyer Flask (E-125)	Corning	431143
Neon™ NxT Disposable 96 Tips & Tubes, 10µL	Invitrogen	N1096K
Neon™ NxT Disposable 96 Tips & Tubes, 100µL	Invitrogen	N10096K
Neon™ NxT Electrolytic E10 Buffer	Invitrogen	A54296-02
Neon™ NxT Electrolytic E100 Buffer	Invitrogen	A54297-02
Neon™ NxT Resuspension R Buffer	Invitrogen	A54298-02

Media Preparation

Transfection Growth Medium (1L)

Add the following components to a 0.2 µm aPES 1L filter unit. Filter by suction once all components are added.

- 40mL GlutaMAX™ Supplement (final concentration – 8mM)
- 1mL Anti-Clumping Agent (final concentration – 1:1000)
- Bring total volume to 1000mL with CD FortiCHO Medium

❖ *Note: Do not add antibiotics to the transfection medium. Antibiotics such as Penicillin-Streptomycin can be optionally supplemented in the medium 24 hours after transfection.*

Standard CleanCut GS CHO Cell Incubation Conditions

Temperature: 37°C

CO_2 level: 8%

Shaking:

- Well plates – Do not shake
- T-25 Flasks – 90 RPM, 19mm Orbit
- E-125 Flasks – 125 RPM, 19mm Orbit

Protocols

Transfection Planning

1. Choose either the 10µL or 100µL reaction scales of Neon transfection and base all calculations on this scale. See table 1 below.

Table 1. Cell Count and Volumes for Neon Transfections

Neon Reaction Scale	Cells Per Transfection	Plate Format	Volume Media Per Well
10µL	$0.2x10^6 – 0.25x10^6$	24-well	0.5mL
100µL	$1x10^6 – 2x10^6$	6-well	2mL

❖ *Note: 10µL reactions require fewer cells and less reagent per transfection. Demeetra recommends using this format for small-scale R&D tests and endpoint experiments where a large final cell volume is not required. For typical cell line development workflows, Demeetra recommends using 100µL reactions for faster cell scale-up.*

2. Calculate volumes of payload (RNA and DNA to be transfected) per reaction based on the selected reaction scale. Demeetra recommends the following conditions:
    a. Transfect 4µg Transposon DNA per $1x10^6$ cells
    b. Use a 4:1 ratio of transposon DNA to Harbor-IN mRNA
    c. Prepare transfection reaction volumes with at least a 10-30% overage to make pipetting easier and to avoid bubbles.
    d. When possible, restrict the total volume of payload to 10% or less of the total reaction volume to maintain consistent transfection conditions.

❖ *Note: Demeetra recommends preparing each Neon reaction in a 1.5mL microfuge tube following this workflow:*
    - *Add DNA and RNA to the tubes on ice*
    - *Bring volume up to a set volume using buffer R*
    - *Add a set volume of cells (resuspended in buffer R) to the reaction mix*
    - *Mix by pipetting*
    - *Transfect*

❖ *Note: High-concentration reagents are generally preferred and can be diluted in buffer R to make pipetting easier.*

3. Determine a set volume of cells (resuspended in buffer R) and the volume of buffer R to add to each tube to maintain the same total volumes for all reactions. See tables 2 and 3 below for recommended setups.

Table 2. Example Harbor-IN Neon Setup using **10µL Reaction Scale with 20% overage**

Reagent and Concentration	Condition 1: Transfection Control	Condition 2: HI 4:1	Condition 3: HI 4:1
Transposon 1 (1µg/µL)	-	1.2 µL (1.2 µg)	-
Transposon 2 (2µg/µL)	-	-	0.6 µL (1.2 µg)
Transposase: Harbor-IN mRNA (1µg/µL)	-	0.3 µL (0.3 µg)	0.3 µL (0.3 µg)
eGFP mRNA (1µg/µL)	0.3 µL (0.3 µg)	-	-
Buffer R	1.7 µL	0.5 µL	1.1 µL
Cells	$0.3x10^6$	$0.3x10^6$	$0.3x10^6$
Cell Volume	10 µL	10 µL	10 µL
Total Volume	12 µL	12 µL	12 µL

Table 3. Example Harbor-IN Neon Setup using **100µL Reaction Scale with 10% overage**

Reagent and Concentration	Condition 1: Transfection Control	Condition 2: HI 4:1	Condition 3: HI 4:1
Transposon 1 (1µg/µL)	-	8.8 µL (8.8 µg)	-
Transposon 2 (2µg/µL)	-	-	4.4 µL (8.8 µg)
Transposase: Harbor-IN mRNA (1µg/µL)	-	2.2 µL (2.2 µg)	2.2 µL (2.2 µg)
eGFP mRNA (1µg/µL)	2.2 µL (2.2 µg)	-	-
Buffer R	8.8 µL	-	4.4 µL
Cells	$2.2x10^6$	$2.2x10^6$	$2.2x10^6$
Cell Volume	99 µL	99 µL	99 µL
Total Volume	110 µL	110 µL	110 µL

Transfection Protocol

1. Seed CleanCut cells at $1x10^6$ cells/mL in 30mL 1 day prior to transfection.
2. Move Harbor-IN and transposon plasmids on ice to thaw and continue with the next steps. Allow 30min – 1hr for thawing and then gently mix by pipetting to ensure reagents are totally thawed.

❖ *Note: Keep Harbor-IN mRNA on ice whenever possible to avoid RNA degradation.*

3. Pre-warm Transfection Growth Medium and aliquot into plates; keep in incubator until transfection.
4. Move the Neon NxT Pipette Station, Neon electrolytic buffer (E10 for 10µL, E100 for 100µL), and resuspension buffer R to room temperature in the biosafety cabinet.
5. Aliquot 2mL of electrolytic buffer into each Neon NxT Tube and secure the Tube in the Neon NxT Pipette Station tube chamber.

❖ *Note: The same tube and electrolytic buffer can be used up to 10 times if transfecting with the same DNA/RNA. Prepare a new tube with fresh buffer for each new DNA/RNA combination to avoid carryover contamination.*

6. Prepare payload tubes on ice by adding DNA, RNA, and buffer R to a 1.5mL tube for each transfection condition. Keep on ice until transfection. (see **Transfection Planning** section above)
7. Count cells to determine the cell density of the suspension.
    a. Gently mix cells in a 50mL conical tube by pipetting 5-7 times with a serological pipet.
    b. Add 20ul of cell suspension to 20ul 0.04% Trypan Blue solution, mix by pipetting up and down 5-7 times.
    c. Add the solution to a slide and count using an automatic cell counter.
    d. Ensure cell viability is greater than 90%.
8. Transfer cells to a 15-mL conical tube.
   (total cells = number of cells per reaction x 2-6 reactions)

❖ *Note: If performing multiple transfections, spinning down enough cells for multiple reactions is ideal to maintain consistent cell numbers across transfections.*

9. Centrifuge the cells at 250 RCF for 5 minutes at room temperature. Do this right before you are ready to electroporate to minimize cell stress.

10. After centrifugation, move the payload tubes you plan to use with these cells to room temperature.
11. Aspirate media off the cell pellet and resuspend the cells in buffer R by pipetting 6-8 times. (buffer R volume = number of reactions spun x set volume of cells per reaction)
12. Immediately transfer the set volume of cells per reaction into 1-6 payload tubes. Do this quickly to ensure each tube gets a consistent density of cells. Electroporation is carried out at room temperature; do not replace tubes on ice after this point.

❖ *Note: Avoid storing cells in Resuspension Buffer R for more than 10 minutes at room temperature. Only work with a number of tubes you feel comfortable transfecting within the 10min period*.

13. Gently mix the cells within the first tube 4-5 times to ensure payload is evenly distributed.
14. Load the Neon pipette according to manufacturer's instructions
15. Press the plunger on the Neon NxT Pipette to the first stop and immerse the tip into the cell-payload mixture.
16. Slowly release the plunger to aspirate the cell-payload mixture into the NEON NxT Tip, avoiding air bubbles as they will cause arcing during electroporation.
17. Dock the Neon NxT Pipette with the sample vertically into the Neon NxT Tube placed in the Neon NxT Pipette Station until you hear a click sound.
18. Ensure that the pipette projection is inserted into the groove of the pipette station, and that the tip is submerged in electrolytic buffer.
19. Press "electroporate" on the Neon NxT device screen using these settings: **1650V, 10ms, 3 pulses**

❖ *Critical Checkpoint: observe the pipet tip during electroporation to ensure there is no arcing or loss of reagent from the pipet tip into the electrolytic buffer. Even if the display says the electroporation was a success, the cells will suffer if there was even a small arcing event. When you see no arcing and the device displays a green checkmark, you are ready to continue.*

20. After successful electroporation, directly add content from NxT tip to the culture plate containing pre-warmed media.
21. Repeat steps 13-20 for all other reactions.
22. Gently rock the plate to evenly distribute the cells and replace the well plate with cells in incubator without shaking.

23. Continue to Demeetra **SOP002, Transfection Recovery and GS Selection SOP**.

Example of CLD workflow using CleanCut GS cells and Harbor-IN

Initial seeding and preparation are the same as above, however, for commercial or scale purposes, here are our recommendations.

1. Prepare enough reagents for 4 replicate transfections, for total volume ~ 440µL
    a. A single transfection is $2.2x10^6$ cells, for 4 transfections, use $8.8x10^6$ cells.
    b. A single transfection is 2.2µg of Harbor-IN mRNA, use 8.8µg.
    c. A single transfection is 8.8µg of transposon, use 35.2µg.
2. Resuspend $8.8x10^6$ cells in X µL
    a. X µL = 440µL – (mRNA + transposon DNA)
    b. Approximately 396µL
3. Add resuspended cells to the mRNA and transposon DNA and mix by pipetting up and down 3-5 times.
4. Take 100µL with the 100µL NxT Tip and perform electroporation. Add to 2mL warmed media in a 6-well plate.
5. Repeat with new 100µL NxT tip for the next three electroporations.
6. For CLD, there will be around $8x10^6$ cells per condition.
7. Optionally, 24-48 hours post transfection, add ½ volume of fresh media to each well.
8. 72-96 hours post-transfection, cells from all wells can be pooled and checked for viability prior to glutamine selection.

❖ *Note: Viability should reach at least 85% before starting selection. We recommend an initial seeding density of $1x10^6$ cells/mL in glutamine selection media. For more details, see **SOP002, Transfection Recovery and GS Selection SOP.***

9. The cells can also be transferred into T-25 or E-125 flasks to be shaken at 90 RPM or 125 RPM, respectively.

Harbor-IN Neon Transfection Workflow