

Pneumatic microplate mixing accelerates CHO single-cell cloning workflows and improves early clone expansion

Feng-Chun Yen · Yi-Ming Chiu · Shih-Pei Lin · Ching-Nan Lin · Wei-Rou Wang · Cheng-Han Tsai

Received: 20 July 2026 / Accepted: 5 October 2026

© The Author(s) 2026

Abstract

Background Stable Chinese hamster ovary (CHO) cell line development depends on efficient recovery and expansion of monoclonal populations after single-cell cloning (SCC). Early-stage clones, however, are typically expanded under static microplate conditions before transfer to suspension culture, creating a physiological discontinuity that can delay adaptation and obscure differences in clone performance. Here, we evaluated C.NEST, a pneumatic microplate mixing system, as a strategy for improving early CHO-K1 clone expansion and facilitating transition to suspension culture.

Results Benchmark studies showed that pneumatic microplate mixing improved culture performance compared with static culture and supported shake-flask-like growth. Operating-window studies in 24-well plates showed higher viable cell densities under pneumatic mixing while generally maintaining high viability, with the response depending on inoculation density and mixing intensity. In clone-level validation studies, pneumatic-mixing pre-culture was associated with faster subsequent suspension adaptation. In an integrated SCC workflow demonstration, pneumatic mixing shortened microplate expansion by 3 days and reached the fed-batch production stage 7 days earlier than the corresponding static workflow. Among the eight clones advanced to production, 96-well relative titer was positively correlated with final fed-batch titer under pneumatic mixing (Spearman's $\rho=0.7857$, $P=0.0279$), while the static workflow showed a weak negative, non-significant correlation.

Conclusions Early pneumatic mixing in microplates improves clone expansion and facilitates transition from static SCC culture to suspension culture, thereby accelerating progression through CHO cell line development. Among the selected clones advanced to production, the observed correlation pattern was consistent with greater preservation of clone ranking under the tested pneumatic-mixing conditions.

Keywords Cell line development · Single-cell cloning · Microplate mixing · Pneumatic agitation · Suspension adaptation

This Article in Press is shared early to give you faster access to new research. It is citable and carries a permanent DOI. The final edited version will replace it automatically.



Background

Chinese hamster ovary (CHO) cells remain the dominant mammalian host for producing recombinant therapeutic proteins because of their adaptability to suspension culture, capacity for complex post-translational modification, and long history of regulatory acceptance [1–3]. In stable cell line development (CLD), single-cell cloning (SCC) is routinely used to isolate monoclonal populations and support downstream requirements for cell-line consistency, productivity, and product quality [4, 5]. Despite its routine use, SCC remains operationally constrained by the slow recovery and expansion of single-cell-derived clones during the earliest stages of development [5, 6].

A typical SCC workflow begins in static high-density microplates and proceeds through sequential expansion into larger-scale culture formats before transfer to shaking suspension systems such as TubeSpin bioreactors or shake flasks [5, 7]. This progression creates a mismatch between the static microenvironment used for early clone recovery and the dynamic suspension conditions used later in development. During this transition, clones often show variable recovery, prolonged lag phases, and inconsistent growth kinetics [5, 7]. These effects can delay scale-up and may also distort clone ranking if poor adaptation obscures the intrinsic productivity of otherwise promising clones [6, 8].

Static microplate culture is vulnerable to local heterogeneity in oxygen availability, nutrient distribution, metabolite accumulation, and evaporation-associated effects [9]. Because these formats provide little convective mixing, early-stage clones may experience microenvironments that differ substantially from those encountered after transfer into suspension culture [7, 9]. Although active microplate and microscale bioreactor systems can improve mass transfer, many rely on vessel-level agitation or are less compatible with standard plate-based handling and imaging workflows [10]. Introducing controlled mixing while preserving conventional microplate formats could therefore improve continuity between early clone expansion and later suspension-based development.

C.NEST is a pneumatic microplate agitation platform for standard microplate formats. A specialized lid generates alternating suction and expulsion cycles that drive within-well medium circulation while the plate remains stationary, thereby avoiding orbital shaking of the plate. This design promotes convective mixing while minimizing splashing, cross-well contamination, and uneven liquid movement, and it remains compatible with routine plate handling, incubation, and monitoring during early-stage clone expansion.

Here, we evaluated whether pneumatic microplate mixing could improve early microplate expansion and reduce the transition between static clone outgrowth and subsequent suspension culture. We first benchmarked a prototype pneumatic mixing system against static plate and shake-flask cultures and then established operating conditions for early 24-well expansion. We further examined clone growth, viability, productivity, suspension adaptation, and alignment with shake-flask performance. Finally, we incorporated pneumatic mixing into an integrated SCC workflow to evaluate its effects on workflow duration and downstream clone performance.

Methods

Cell lines and maintenance culture

Suspension-adapted CHO-K1 and monoclonal antibody-expressing CHO-K1Q cell lines (QuaCell Biotechnology) were used in this study. Both cell lines were maintained in 125 mL shake flasks (Thermo Fisher Scientific, 4115–0125) containing EX-CELL CD CHO Fusion medium (Merck, 14365 C) at a 20 mL working volume. Cultures were maintained at 37 °C, 5% CO₂, and 150 rpm on an orbital shaker. Routine passaging was performed every 3–4 days by reseeding cells at 3×10^5 cells/mL. Viable cell density and viability were measured using 0.4% Trypan Blue Stain (Gibco, 15250-061) with a FACSCOPE B Cell Counter (Curiosis, South Korea).

Pneumatic microplate mixing system and culture conditions

Pneumatic microplate mixing was performed using the C.NEST system (Leadgene Biosolutions, Taiwan) with standard 96-well (Corning, 3599) and 24-well (Greiner Bio-One, 662102) plates fitted with the corresponding pneumatic mixing lids (Leadgene Biosolutions, Taiwan). The system comprises four independently operated incubation chambers equipped with temperature, humidity, and CO₂ monitoring and UV light for chamber sterilization (Fig. 1a, b). The pneumatic lid contains airflow ports, a lid chamber, and fluidic structures that transmit pneumatic pressure changes into individual wells (Fig. 1c and Supplementary Table 1). During the suction phase, liquid is drawn upward through the fluidic structure, whereas during the expulsion phase, liquid is driven downward and redistributed within the well (Fig. 1d). Repeated cycling promotes vertical medium circulation without orbital movement of the plate. The plate-lid assembly was placed on the plate holder together with a sterile water reservoir to maintain humidity during culture (Fig. 1e).

Cultures were maintained at 37 °C and 5% CO₂. Working volumes were 200 µL per well for 96-well plates and 1,400 µL per well for 24-well plates. Static control plates were maintained in a humidified incubator under the

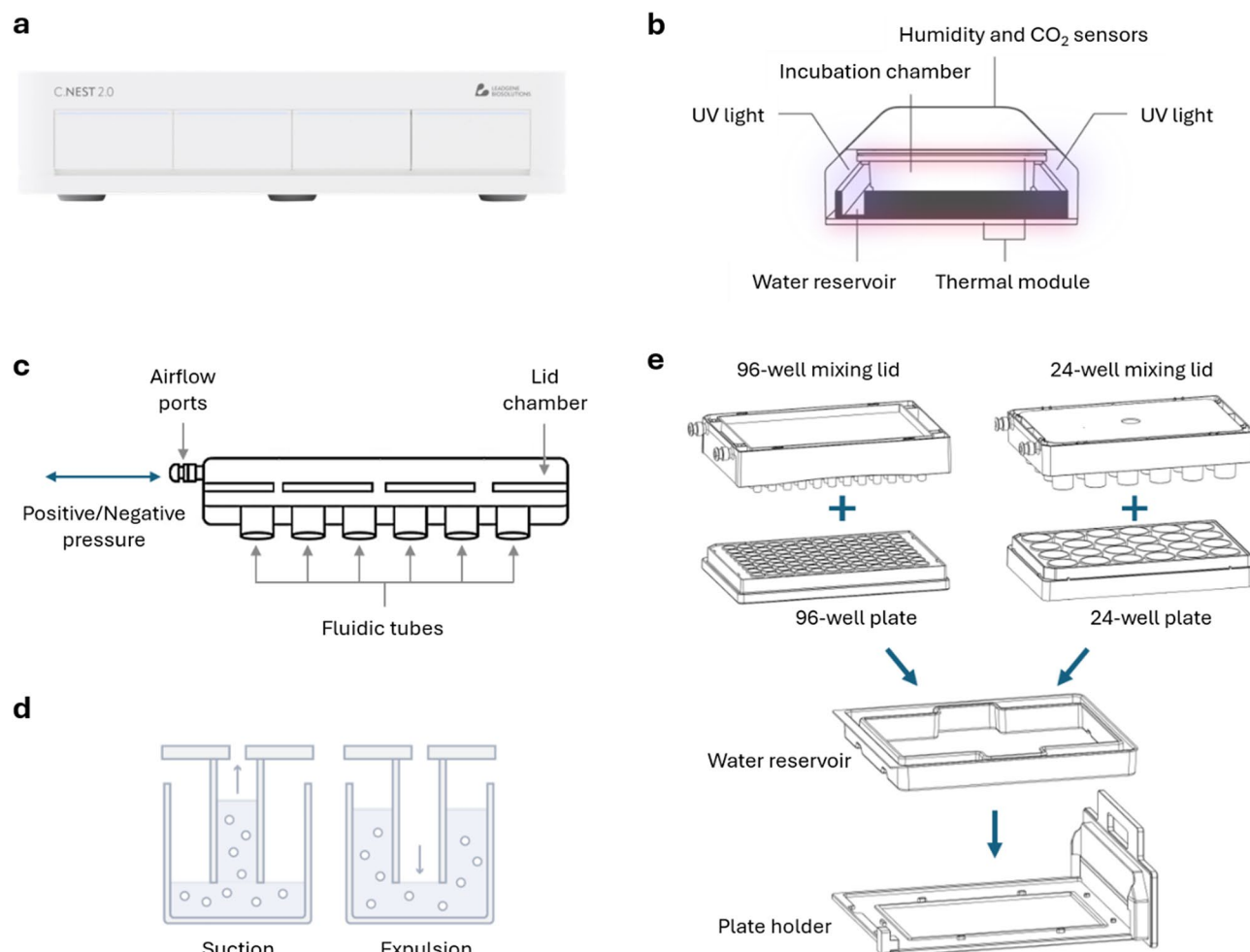


Fig. 1 Overview of the C.NEST pneumatic microplate mixing culture system. **(a)** C.NEST system. **(b)** Scheme of the incubation chamber and environmental control components. **(c)** C.NEST pneumatic lid design showing airflow ports, a lid chamber, and fluidic tube structures. **(d)** Illustration of pneumatic suction and expulsion phases that generate vertical medium circulation within each well. **(e)** Scheme showing the lid-plate assembly and its placement on a plate holder with a sterile water reservoir to maintain humidity during culture

same environmental conditions. Plate and chamber positions were randomized among comparative conditions to minimize potential positional bias.

The tested mixing-cycle settings were selected to span the operational range of pneumatic mixing intensities evaluated for the respective plate formats. For subsequent workflow studies, 50 s/cycle was used for 96-well expansion as a comparatively gentle mixing condition, whereas 10 s/cycle was used for 24-well expansion based on the operating-window experiments, which showed enhanced cell expansion while generally maintaining high viability. These settings represent operating conditions established for the cell lines, working volumes, and culture conditions evaluated in this study and should not be interpreted as universally optimal across all cell densities, media formulations, or process conditions. During routine culture monitoring, no visible foaming or persistent bubble accumulation was observed in either 96-well or 24-well pneumatic-mixing cultures under the operating conditions used in this study.

Viable cell density and viability were monitored every 3–4 days. For benchmark experiments, doubling time and protein fold change were calculated according to Eqs. 1 and 2, respectively.

$$\text{Doubling time} = \frac{t_2 - t_1}{\log_2 \left(\frac{N_2}{N_1} \right)} \quad (1)$$

(N_1 and N_2 are the viable cell densities at time points t_1 and t_2 , respectively.)

$$\text{Fold change} = \frac{P_2}{P_1} \quad (2)$$

(P_1 and P_2 are the protein yields on day 1 and at a certain time point, respectively.)

$k_L a$ determination

$k_L a$ was determined by the gassing-out method using the C.NEST system configured for dissolved oxygen monitoring [11]. Briefly, culture medium was added to the dissolved oxygen sensing plate (Leadgene Biosolutions, Taiwan), which was then fitted with the corresponding mixing lid and mixed until dissolved oxygen saturation was reached. The dissolved oxygen signal was calibrated to 100%, after which oxygen was depleted by nitrogen sparging and then restored by switching back to air. Dissolved oxygen was recorded over time during the reoxygenation phase at defined time intervals. The instantaneous rate of oxygen transfer was described by Eq. 3.

$$\frac{dC_L}{dt} = k_L a (100 - C_L) \quad (3)$$

(C_L is the local dissolved oxygen concentration at time t , and the saturated dissolved oxygen concentration is normalized to 100%. k_L represents the mass transfer coefficient, and a represents the gas-liquid exchange area per unit of liquid volume.)

Assuming constant $k_L a$ and a constant saturated dissolved oxygen concentration, Eq. 3 is integrated to give Eq. 4. $k_L a$ is then determined from the linearized oxygen-transfer relationship during the reoxygenation phase.

$$k_L a (t_2 - t_1) = \ln \left(\frac{100 - C_{L1}}{100 - C_{L2}} \right) \quad (4)$$

(C_{L1} and C_{L2} are the local dissolved oxygen concentrations at time points t_1 and t_2 , respectively.)

Assessment of evaporation-associated volume loss and edge effects

Evaporation-associated edge effects were evaluated in 96-well and 24-well plates containing sterile PBS (200 and 1,400 μL per well, respectively) over a 3-day incubation under either a standard incubator or pneumatic-mixing

conditions. For pneumatic-mixing culture, 96-well plates were operated at 50 s/cycle and 24-well plates at 10 s/cycle, with a sterile water reservoir placed in the chamber to maintain humidity. Evaporation was quantified from the change in liquid volume relative to the initial volume according to Eq. 5. Wells were classified as corner, edge, or center wells, and percentage volume loss after 3 days was compared between standard incubator and pneumatic-mixing conditions within each positional group. PBS was used as a defined, cell-free test solution to isolate evaporation-associated volume loss from cell-dependent or medium-composition effects during the short-duration plate-culture interval relevant to the workflow.

$$\text{Evaporation (\%)} = \frac{V_0 - V_t}{V_0} \times 100 \quad (5)$$

(V_0 is the initial liquid volume, and V_t is the liquid volume after incubation time t .)

Evaluation of cross-well liquid transfer during pneumatic mixing

Cross-well liquid transfer was evaluated in a 24-well plate using a checkerboard layout of alternating wells containing 1,400 μL of either ddH₂O or 0.2% Trypan Blue solution. Plates were cultured in C.NEST with a pneumatic mixing lid at 37 °C and 5% CO₂ under continuous mixing at 10 s/cycle. They were assessed under two conditions: repeated manual lid opening during a 3-hour culture period, with the lid opened at 15-min intervals, and long-term continuous mixing for 14 days. For OD₅₉₅ measurement, 200 μL of solution was collected from each well and from the moat. Each sampled well was measured in duplicate, and each 96-well readout plate was scanned three times. A Trypan Blue dilution standard curve was generated in parallel and used to define the detection threshold for dye transfer. Corrected OD₅₉₅ values were obtained by subtracting the ddH₂O blank from each measurement.

Single-cell seeding and early clone expansion

Single-cell seeding was performed using the UP.SIGHT system (CYTENA, Germany), enabling automated cell dispensing and image-based monoclonality confirmation [12]. Cells were dispensed into 384-well plates (Corning, 3680) containing 50 μL Cellvento® 4CHO-C Cloning Medium (Merck, 14390 C) per well. Plates were incubated under static conditions at 37 °C and 5% CO₂, and clone outgrowth was monitored by confluency using the CloneSelect Imager (Molecular Devices, USA).

Single-cell deposition and image-based monoclonality confirmation were completed at the 384-well stage before pneumatic mixing was introduced. Clones remained under static conditions during initial single-cell outgrowth, and pneumatic mixing was applied only after established single-cell-derived populations were transferred to the 96-well expansion stage. Accordingly, subsequent mixing and expansion did not alter the documented evidence supporting single-cell derivation.

TubeSpin adaptation culture

In validation studies, 96 clones per workflow were screened in 96-well plates, and higher-titer clones were advanced at subsequent stages, with 24 clones selected for 24-well expansion and the top 8 clones by relative titer transferred to TubeSpin bioreactors (TPP, 87050) at a 10 mL working volume for suspension adaptation. Cultures were maintained in EX-CELL CD CHO Fusion medium at 37 °C and 5% CO₂ with agitation at 150 rpm on an orbital shaker. Viable cell density and viability were measured 3, 7, and 10 days after transfer to TubeSpin culture, corresponding to workflow days 24, 28, and 31.

Suspension adaptation and antibody production

The integrated SCC workflow was evaluated using a single recombinant production model, anti-PD-L1-expressing CHO-K1Q cells.

After 12 days of static culture following single-cell seeding in 384-well plates, wells exceeding 30% confluency were ranked by relative titer. The top 192 clones were allocated between the two workflow arms, with 96 clones entering static 96-well screening and 96 entering pneumatic-mixing 96-well screening. Identical advancement criteria were applied independently within each arm: 24 higher-titer clones were selected for 24-well expansion, followed by advancement of the top 8 clones by relative titer to suspension adaptation and fed-batch production in 125 mL shake flasks. No randomly selected or deliberately lower-performing clones were followed through the complete workflow. Thus, the fed-batch population was progressively enriched for clones exhibiting higher relative titer at preceding stages. During suspension adaptation, cultures were maintained in EX-CELL CD CHO Fusion medium at 37 °C and 5% CO₂ with agitation at 150 rpm on an orbital shaker. For the antibody production phase, cells were cultured for 14 days in Dynamis AGT Medium (Gibco, A26175-01) supplemented with 1% (v/v) Anti-Clumping Agent (Gibco, 01-0057DG) and maintained at 37 °C and 8% CO₂ under the same agitation conditions. Cell Boost 7a/7b solutions (Cytiva, SH31026.01 and SH31027.01, respectively) were added according to the manufacturer's instructions. Glucose solution (200 g/L in ddH₂O; Merck, G7021) was supplemented as needed to maintain an adequate carbon source.

Anti-Clumping Agent was used only during shake-flask fed-batch production and was not added during 96-well or 24-well pneumatic-mixing culture. Its inclusion was part of the fed-batch production protocol and was not introduced in response to foaming, aggregation, or cell clumping observed during the preceding microplate-expansion stages.

Antibody titer measurement

Titers of anti-PD-L1 antibody were measured using the F.QUANT Fc Titer Assay (CYTENA, Germany) according to the manufacturer's instructions. Culture supernatants were loaded onto Fc-specific assay plates, and fluorescence intensity was measured to obtain F.QUANT single values. For validation studies and other intermediate workflow assessments, these values were used as relative titer readouts to compare clone performance. For the benchmark experiments and for samples collected at the end of fed-batch culture, absolute titer (mg/L) was calculated using a standard curve generated from serial dilutions of an anti-PD-L1 antibody stock solution (1 mg/mL) and reported as the final antibody concentration. Cell-specific productivity (qP; pg/cell/day) was calculated from measured absolute titer values according to Eq. 6.

$$qP = \frac{P_2 - P_1}{IVCC}, \quad IVCC \approx \frac{X_{v1} + X_{v2}}{2}(t_2 - t_1) \quad (6)$$

(P₁ and P₂ are the antibody concentrations at time points t₁ and t₂, respectively. The integral of viable cell concentration (IVCC) was estimated by the trapezoidal method, where X_{v1} and X_{v2} are viable cell densities at the corresponding time points.)

Statistical analysis

All statistical analyses were performed using GraphPad Prism version 8.0.1 (GraphPad Software, USA). Experimental replication was defined according to the design of each study. Benchmark experiments were performed as three independent experimental runs using separately prepared cultures in separate culture vessels or plates, and one-way ANOVA followed by Tukey's multiple-comparisons test was used.

For the 24-well operating-window experiments shown in Fig. 3 and Supplementary Fig. 4, each mixing condition was evaluated on a separate plate containing all tested initial seeding densities, with four replicate wells per

seeding density. Two-way ANOVA was performed using initial seeding density and mixing condition as factors, followed by Dunnett's multiple-comparisons test comparing each pneumatic-mixing condition with the static control within each seeding density. Because each mixing condition was represented by one plate, these analyses reflect well-level variability and do not account for between-plate variability.

For clone-rank analysis, Spearman rank correlation was used to assess the association between early-stage relative titer and final fed-batch titer among the eight clones advanced to production in each workflow. Figures 4 and 5 represent clone-level observations from one SCC campaign; individual clones were therefore not interpreted as independent campaign-level biological replicates. Data are presented as mean $\pm$ s.d. where appropriate. Statistical significance was defined as follows: *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; ****: $P < 0.0001$.

Results

Characterization of oxygen transfer and operational performance during pneumatic microplate mixing

We first evaluated oxygen-transfer performance across plate formats, working volumes, and pneumatic mixing-cycle settings to define operating conditions for microplate culture. The volumetric oxygen mass transfer coefficient, k_La , is a key parameter for characterizing oxygen transfer from the gas phase to the liquid phase and is commonly used in bioprocess scale-up and culture-condition optimization [13]. In microplate systems, mixing intensity, working volume, airflow characteristics, and medium composition can all influence k_La [9]. Because mixing cycle time is a directly adjustable operating parameter of the pneumatic mixing system, we assessed four settings: 5, 10, 30, and 50 s per cycle. Each cycle consisted of a suction phase followed by an expulsion phase and was repeated continuously during operation. The 50 s/cycle condition represented the gentlest setting, comprising 25 s of suction and 25 s of expulsion, whereas shorter cycle times produced more frequent mixing. These settings corresponded to reciprocal mixing rates ranging from 0.20 to 0.02 s^{-1} .

Across both 96- and 24-well formats, k_La increased with increasing mixing rate, indicating that more frequent pneumatic cycling enhanced oxygen-transfer capacity (Table 1). This trend was observed across the tested plate formats. At a given mixing rate, higher working volumes also produced higher k_La values under the tested conditions (Table 1). Together, these results show that oxygen-transfer performance can be modulated by adjusting mixing cycle time and working volume, providing a practical basis for selecting operating conditions for subsequent clone-expansion experiments.

Because pneumatic mixing increases medium movement and gas exchange relative to conventional static incubation, we next evaluated evaporation-associated volume loss and positional edge effects. Wells were grouped as corner, edge, or center wells and compared between a standard humidified incubator and the pneumatic-mixing system. After 3 days, percentage volume loss in 96-well plates did not differ significantly between conditions across corner, edge, or center regions (Supplementary Fig. 1a). In 24-well plates, percentage volume loss in corner and center wells was likewise similar between conditions. Edge wells showed greater cumulative volume loss under pneumatic mixing than in the standard incubator ($3.4 \pm 0.5\%$ versus $2.3 \pm 0.3\%$, respectively;

Table 1 k_La comparison across C.NEST well formats and working volumes

Mixing speed (s/cycle)	Mixing rate (s^{-1})	24-well plate		96-well plate
		1.4 mL	1.0 mL	0.2 mL
5	0.20	8.40	7.98	—
10	0.10	5.21	3.00	2.67
30	0.03	2.69	2.25	1.85
50	0.02	2.04	1.58	1.31

*—, not tested; 0.2 mL was outside the working volume range evaluated for the 24-well plate

Supplementary Fig. 1b). Because the 3-day assessment was designed to reflect the short-duration pneumatic-mixing stages used in the present workflow, these data indicate that evaporation-associated volume loss remained limited over the relevant operating interval, while also highlighting the need to monitor edge wells.

We also evaluated whether pneumatic mixing could introduce cross-well or well-to-moat liquid transfer. In a supplementary dye-transfer assay, corrected OD₅₉₅ values from dye-free wells and moat samples remained below the detection threshold during repeated lid opening and during 14 days of continuous mixing (Supplementary Fig. 2), supporting preservation of well isolation under the tested operating conditions.

Prototype pneumatic mixing benchmarks against static and shake-flask cultures

To benchmark the effect of pneumatic microplate mixing on culture performance before applying the C.NEST platform to clone-development workflows, we first evaluated a prototype mixing system based on the same pneumatic mixing principle [14]. The prototype pneumatic mixing system was evaluated in a 24-well microplate format and compared in parallel with static 24-well microplate culture and conventional shake-flask culture as alternative formats for early cell expansion (Fig. 2a). Over 3 days of culture, the pneumatic mixing condition supported progressively greater cell growth than static plate culture (Fig. 2b). By day 3, its expansion profile approached that of shake-flask culture and yielded significantly higher viable cell numbers than static plate culture (Fig. 2b). Endpoint analysis showed no statistically significant differences between the pneumatic mixing condition and shake-flask culture in doubling time or protein yield, whereas both conditions outperformed static

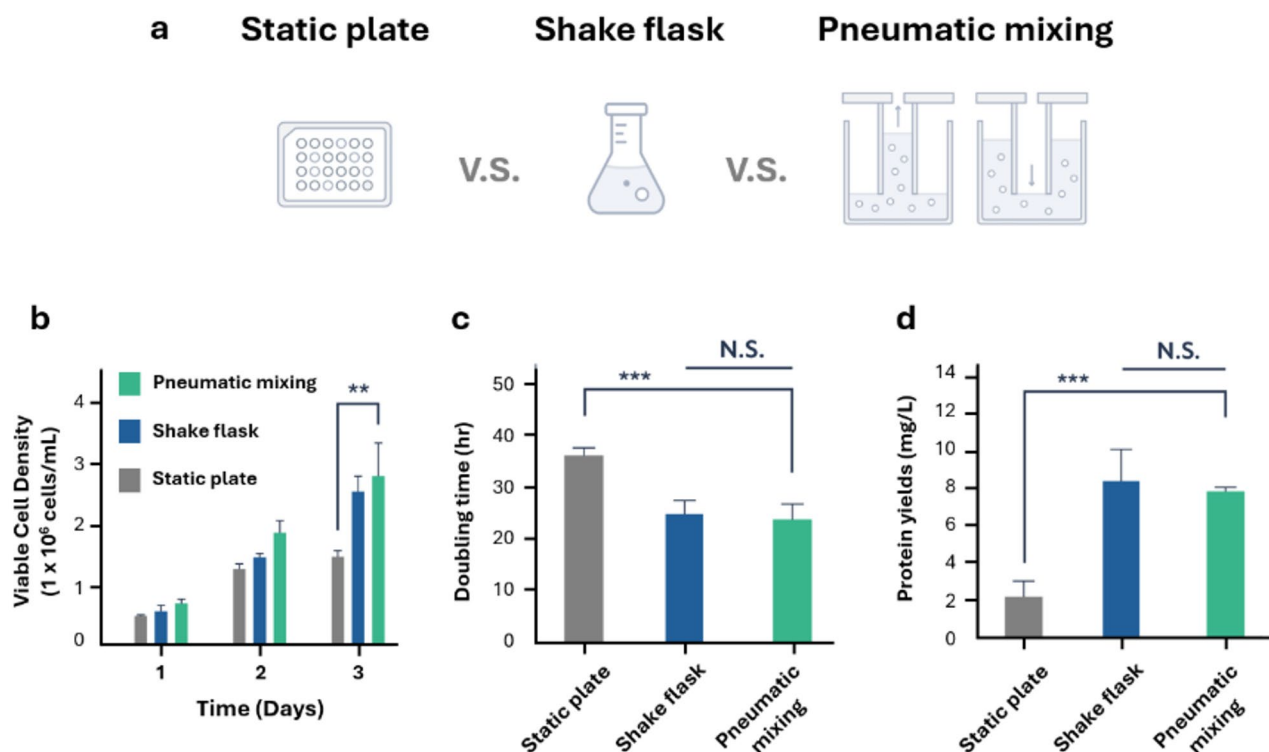


Fig. 2 Benchmarking of pneumatic microplate mixing against static plate and shake-flask culture. **(a)** Experimental design for comparison of the pneumatic mixing condition with static microplate culture and conventional shake-flask culture. **(b)** Viable cell expansion under the pneumatic mixing condition, shake-flask culture, and static plate culture over 3 days. **(c, d)** Doubling time **(c)** and protein yield **(d)** measured at the end of the culture period. Data are presented as mean $\pm$ s.d. ($n=3$ independent experiments). N.S.: not statistically significant. **: $P<0.01$; ***: $P<0.001$. Statistical comparisons were performed by one-way ANOVA followed by Tukey's multiple-comparisons test

plate culture (Fig. 2c, d). These data show that pneumatic microplate mixing can support culture performance comparable to shake-flask culture during early expansion.

We next performed the same parallel comparison in a 96-well microplate format over 5 days of culture. Viable cell density under the pneumatic mixing condition was comparable to that in shake-flask culture, with both conditions significantly exceeding static plate culture (Supplementary Fig. 3a). Viability remained above 80% in both the pneumatic mixing condition and shake-flask culture at the end of culture, whereas static plate culture declined to approximately 60% (Supplementary Fig. 3b). Doubling time and fold change in protein yield likewise showed superior performance of the pneumatic mixing condition and shake-flask culture relative to static plate culture (Supplementary Fig. 3c, d). Together, these results indicate that pneumatic mixing can confer suspension-like growth characteristics across both 24-well and 96-well microplate formats. Based on these benchmarking results, subsequent experiments were conducted with the finalized C.NEST system to optimize mixing conditions and assess its use in clone expansion, TubeSpin adaptation, and accelerated SCC workflows.

Pneumatic mixing improves early 24-well expansion and defines an operating window

To define practical operating conditions for introducing pneumatic mixing during early clone expansion, we compared 24-well cultures under static conditions and pneumatic mixing at 50, 25, or 10 s per cycle across a range of initial seeding densities. After 3 days of culture, viable cell densities were similar among conditions at the two lowest seeding densities, whereas all three pneumatic-mixing conditions showed higher mean viable cell densities than static culture from 1×10^5 cells/mL upward (Fig. 3a). The separation became more pronounced at higher inoculation densities, and the 10 s/cycle condition produced the highest mean viable cell densities at 5×10^5 and 1×10^6 cells/mL. At 1×10^6 cells/mL, the 10 s/cycle condition increased the Day 3 VCD from 1.87×10^6 to 3.70×10^6 cells/mL, corresponding to an approximately 98% increase relative to static culture. Cell viability remained generally high across conditions. At the highest initial seeding density, the gentler 50 and 25 s/cycle conditions maintained higher mean viability than static culture, whereas viability under the 10 s/cycle condition was comparable to the static condition (Fig. 3b).

A similar comparison extended to day 4 showed that pneumatic mixing continued to support higher viable cell densities than static culture, particularly from 1×10^5 cells/mL upward (Supplementary Fig. 4a). At the highest inoculation densities, however, stronger mixing was accompanied by a modest reduction in viability, most notably under the 10 s/cycle condition (Supplementary Fig. 4b). Together, these findings indicate that the benefit of

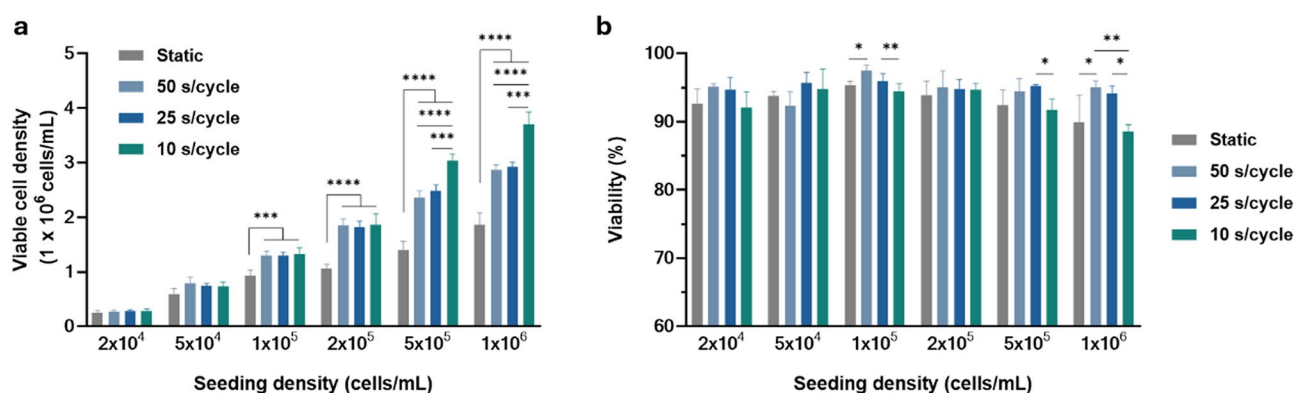


Fig. 3 Effect of pneumatic mixing on 24-well culture performance across initial seeding densities. **(a)** Viable cell density after 3 days of culture under static conditions or pneumatic mixing at 50, 25, or 10 s per cycle. **(b)** Cell viability under the same conditions. Data are presented as mean $\pm$ s.d. of four replicate wells per seeding density. Statistical comparisons were performed using two-way ANOVA with initial seeding density and mixing condition as factors, followed by Dunnett's multiple-comparisons test comparing each pneumatic-mixing condition with the static control within each seeding density. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$

pneumatic mixing depends on both inoculation density and culture duration and support the use of tunable mixing conditions during early clone expansion.

To further assess how closely pneumatic-mixing culture recapitulated conventional suspension culture, we compared culture performance in pneumatic mixing and shake flasks using viable cell density and antibody titer as readouts. Pneumatic-mixing and shake-flask cultures showed a positive correlation for both viable cell density and antibody titer, with coefficients of determination of $R^2=0.7563$ and $R^2=0.9298$, respectively (Supplementary Fig. 5a, b). Because these correlations were based on a limited number of tested conditions, the resulting coefficients should be interpreted as preliminary indicators of cross-format association rather than precise estimates of predictive performance.

Pneumatic mixing enhances clone expansion and subsequent TubeSpin adaptation

Having established that pneumatic mixing supports early expansion, we next investigated whether these benefits could improve downstream single-cell cloning (SCC) workflows. Clones derived from 384-well static cultures were advanced through either a conventional static workflow or a pneumatic-mixing workflow before TubeSpin suspension adaptation (Fig. 4a). In both cases, clones were first cultured for 4 days in 96-well plates and then for 3 days in 24-well plates under their respective static or mixing conditions, after which they were transferred to TubeSpin on day 21 for suspension adaptation (Fig. 4a). In each workflow, 96 clones entered 96-well screening, from which higher-titer clones were advanced at each subsequent step, with 24 clones selected for 24-well expansion and the top 8 clones by relative titer advanced to TubeSpin adaptation.

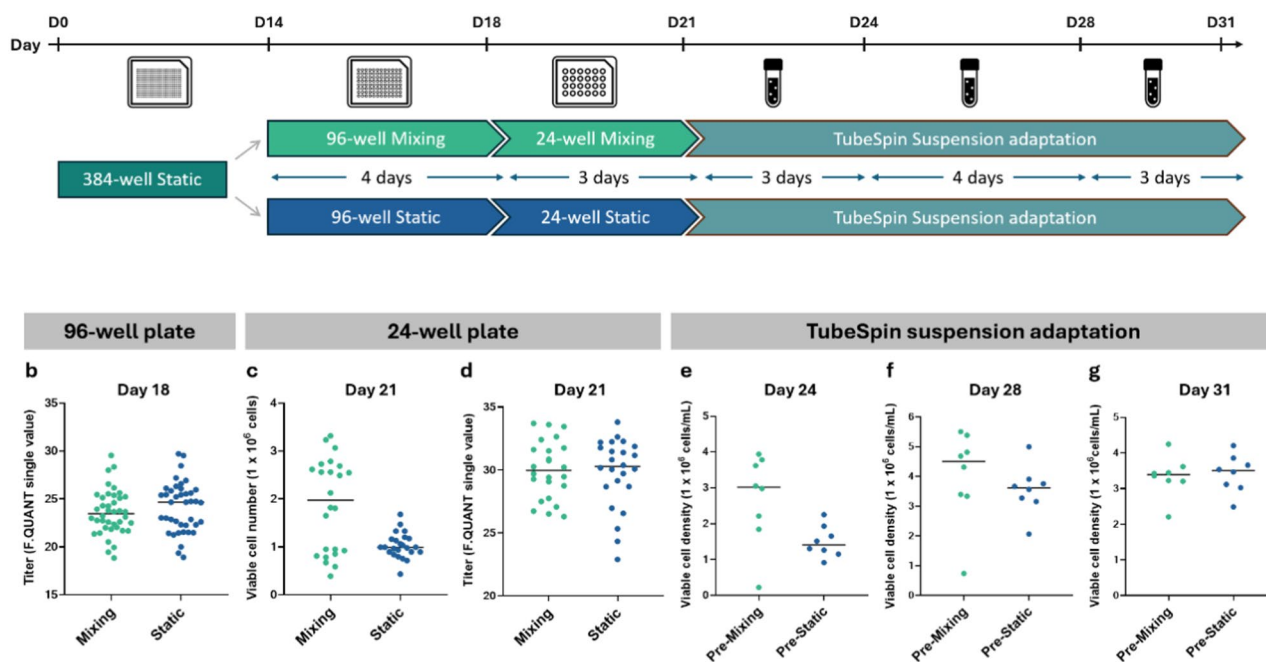


Fig. 4 Pneumatic-mixing clone expansion improves subsequent TubeSpin adaptation. **(a)** Scheme of the SCC workflow. Clones derived from 384-well static cultures were advanced through either a conventional static workflow or a pneumatic-mixing workflow before TubeSpin suspension adaptation. Higher-titer clones were selected at each stage, with 96 clones entering 96-well screening, 24 advancing to 24-well expansion, and the top 8 transferred to TubeSpin adaptation. **(b)** Relative titer distribution after 4 days of culture in 96-well plates under static or pneumatic-mixing conditions. **(c, d)** Viable cell number **(c)** and relative titer distribution **(d)** after an additional 3 days of culture in 24-well plates. **(e-g)** Viable cell densities on days 24 **(e)**, 28 **(f)**, and 31 **(g)** during subsequent TubeSpin adaptation. Each dot represents an individual clone within the indicated SCC workflow. For TubeSpin adaptation, $n=8$ selected clones per workflow, and the individual clones originate from the same workflow campaign. Titer values in **(b)** and **(d)** represent relative F.QUANT readouts used for clone ranking

After 4 days in 96-well plates, static and pneumatic-mixing conditions showed similar relative titer distributions (Fig. 4b). Following an additional 3 days in 24-well plates, clones expanded under pneumatic mixing reached higher viable cell densities than static-derived clones while maintaining comparable relative titer distributions (Fig. 4c, d). Thus, pneumatic mixing improved clone expansion without broadly compromising antibody productivity. After transferring to TubeSpin culture, clones from the pneumatic-mixing workflow recovered more rapidly and reached higher viable cell densities than clones from static pre-culture (Fig. 4e-g). Viability also recovered more quickly in the pneumatic-mixing workflow than in static culture (Supplementary Fig. 6). Although static-derived clones eventually recovered after additional passages, their delayed adaptation indicates that the transition from static to suspension culture imposes a greater recovery burden.

Integrated pneumatic-mixing SCC workflow accelerates progression to fed-batch production

After verifying that pneumatic mixing supports early clone expansion and facilitates suspension adaptation, we incorporated it into an integrated SCC workflow to assess downstream antibody production (Fig. 5a). After 12 days of static culture following single-cell seeding in 384-well plates, wells exceeding 30% confluency were ranked by relative titer, and the top 192 clones were transferred into either 96-well static culture or 96-well pneumatic mixing culture. In each workflow, 96 clones entered 96-well screening, and the higher-titer clones were advanced at each subsequent step: 24 clones to 24-well expansion, and the top 8 clones by relative titer were advanced to suspension adaptation and fed-batch production.

After 4 days in 96-well plates, clones in the pneumatic-mixing workflow showed a broader distribution of relative titer values, including several high-performing clones (Fig. 5b). Following an additional 3 days in 24-well plates, clones expanded under pneumatic mixing showed higher viable cell densities and relative antibody-titer values than clones in the static workflow (Fig. 5c). Static-derived clones required an additional 3-day expansion in 6-well plates to increase viable cell density sufficiently for subsequent suspension transfer, whereas clones expanded under pneumatic mixing reached the required expansion level after the 24-well stage and progressed directly to shake-flask adaptation.

Following suspension adaptation, both groups were advanced to fed-batch antibody production. Four clones in the pneumatic-mixing workflow achieved final fed-batch titers above the highest value observed in the static workflow (Fig. 5d), and the same four clones also showed cell-specific productivity (qP) values above the highest value observed in the static workflow (Fig. 5e). In addition, three pneumatic-mixing clones consistently remained among the highest-ranked performers across the 96-well, 24-well, and fed-batch stages (Fig. 5b-d), indicating preservation of relative clone ranking across successive stages. In this single paired SCC workflow demonstration, the pneumatic-mixing workflow progressed to suspension adaptation 3 days earlier and reached the fed-batch production stage 7 days earlier than the corresponding static workflow.

To quantify preservation of clone ranking, Spearman rank correlation was calculated between early-stage relative titer and final fed-batch titer among the eight clones advanced to production. In the pneumatic-mixing workflow, 96-well relative titer showed a strong positive correlation with final fed-batch titer (Spearman's $\rho = 0.7857$, $P = 0.0279$, $n = 8$), whereas the static workflow showed a weak negative, non-significant correlation ($\rho = -0.4286$, $P = 0.2992$, $n = 8$). At the 24-well stage, the pneumatic-mixing workflow showed a positive association with final fed-batch titer that did not reach statistical significance ($\rho = 0.7143$, $P = 0.0576$, $n = 8$), whereas the static workflow showed a weak negative, non-significant correlation ($\rho = -0.2143$, $P = 0.6191$, $n = 8$).

Discussion

Early clone expansion remains a practical bottleneck in CHO cell line development because the static microplate conditions used during single-cell cloning differ substantially from the dynamic suspension environments required during later scale-up and production [5, 6, 15, 16]. In this study, pneumatic microplate mixing improved

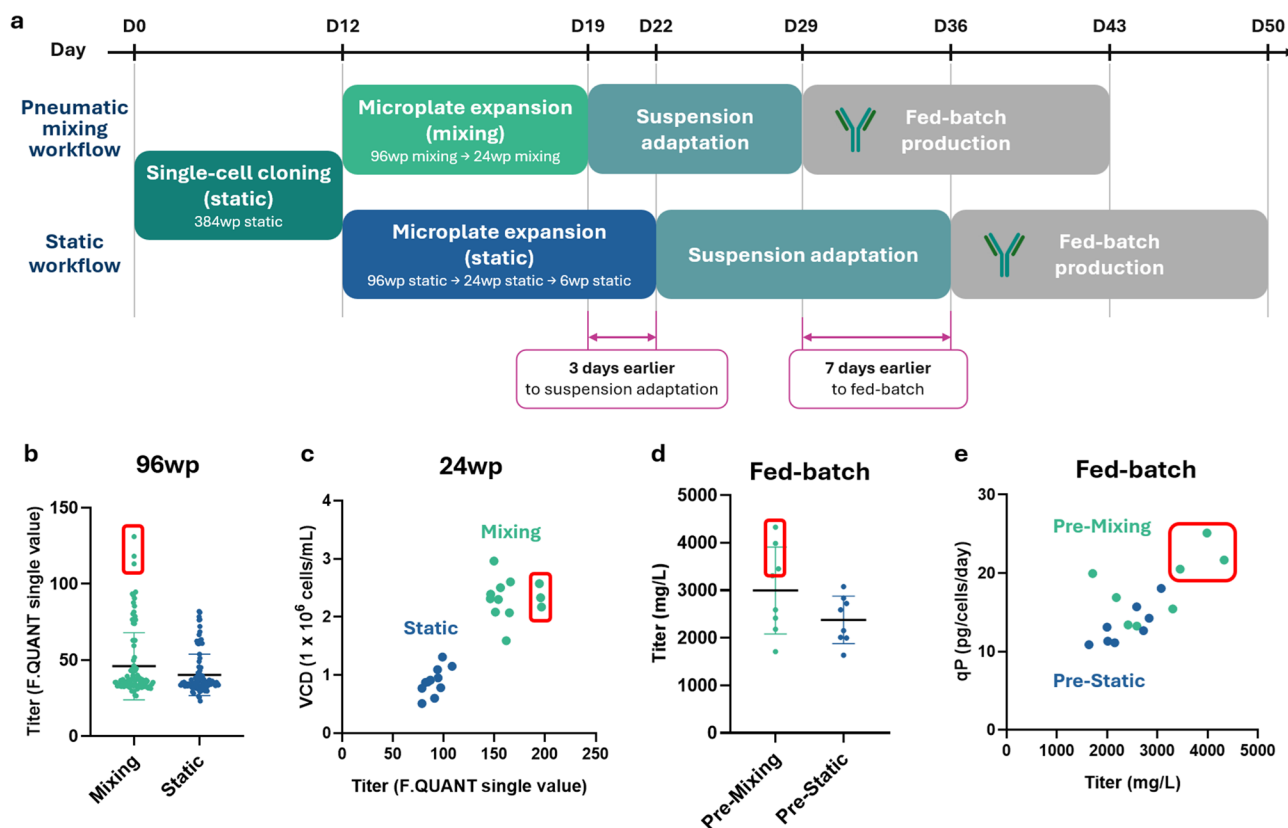


Fig. 5 Integrated pneumatic-mixing SCC workflow improves clone expansion and downstream antibody production. **(a)** Scheme of the integrated SCC workflow comparing pneumatic-mixing expansion with conventional static expansion. After single-cell cloning in 384-well plates, clones were advanced through 96-well and 24-well screening based on relative titer, with 96 clones entering 96-well screening, 24 advancing to 24-well expansion, and the top 8 advanced to suspension adaptation and fed-batch production. Pneumatic mixing cultures progressed directly from 24-well mixing culture to shake-flask adaptation, whereas static cultures underwent an additional 6-well stage before suspension adaptation. **(b)** Relative titer of clones after 4 days of culture in 96-well plates under static or pneumatic-mixing conditions. **(c)** Viable cell density and relative antibody titer after an additional 3 days of culture in 24-well plates. **(d, e)** Final fed-batch titer **(d)** and cell-specific productivity (qP; **e**) after 14 days of production culture. Clones highlighted in the red box denote the three clones that consistently remained among the highest-ranked performers across the 96-well, 24-well, and fed-batch stages. Titer values in **(b)** and **(c)** represent relative F.QUANT readouts used for clone ranking; final fed-batch titer in panel **d** represents absolute antibody concentration (mg/L). Each dot represents one individual clone within the single SCC workflow campaign

early expansion, facilitated subsequent suspension adaptation, and shortened progression through the tested SCC workflow while remaining compatible with standard microplate formats. These findings support the use of controlled early-stage mixing as a workflow-engineering strategy for reducing the transition between static clone outgrowth and later suspension culture.

The benchmark and engineering-characterization experiments established that pneumatic mixing could support growth behavior approaching that observed in shake-flask culture while providing tunable oxygen-transfer performance in both 96- and 24-well formats. The k_La measurements showed that oxygen-transfer capacity could be modulated through mixing-cycle time and working volume. However, the present experiments were not designed to determine whether static cultures were oxygen-transfer limited or whether increased oxygen transfer was the principal mechanism underlying improved expansion. Pneumatic mixing may simultaneously influence oxygen transfer, nutrient and metabolite distribution, cell suspension, and microscale concentration gradients, and the relative contribution of these effects remains to be determined.

The operational assessments also supported the feasibility of applying pneumatic mixing during the short microplate-expansion stages used in this study. The evaporation experiment was intentionally conducted over 3 days because the pneumatic-mixing stages in the workflow lasted approximately 3–4 days. PBS was used as a defined, cell-free surrogate to isolate physical volume loss associated with the incubation and mixing environment. Under these conditions, cumulative volume loss remained limited, although 24-well edge wells showed somewhat greater evaporation than static-incubator controls. Absolute evaporation may nevertheless vary with medium formulation and should be reassessed for substantially longer culture durations or different media systems. Similarly, corrected OD₅₉₅ values remained below the detection threshold during both repeated lid opening and 14 days of continuous mixing, supporting preservation of well isolation under the tested conditions.

No visible foaming or persistent bubble accumulation was observed during routine 96- or 24-well pneumatic-mixing culture, and cell viability remained generally high. Nevertheless, dedicated quantitative measurements of air-liquid interfacial stress, protein aggregation, or shear-associated cell damage were not performed. These parameters should therefore be examined in future studies, particularly for extended operation, alternative medium formulations, or more shear-sensitive cell lines.

The operating-window experiments further showed that the biological response depended on inoculation density, culture duration, and mixing intensity. Pneumatic mixing increased viable cell density most clearly at intermediate-to-high starting densities, whereas stronger mixing at the highest density and longer culture duration was associated with modest reductions in viability. These findings support the use of tunable mixing conditions rather than a single universally optimal setting. The observed growth and titer trends also showed positive association with shake-flask performance, although these correlations were based on a limited number of tested conditions and should therefore be interpreted as preliminary indicators of cross-format alignment rather than precise estimates of predictive performance.

The present comparisons demonstrate a benefit of introducing controlled mixing relative to static microplate culture, but they do not establish that the biological effect is unique to pneumatic actuation. Part of the observed advantage may arise more generally from maintaining cells in a dynamic, suspension-compatible environment before transfer to conventional suspension culture. This distinction is important because alternative microscale bioreactors and agitated microplate systems also improve mixing and mass transfer [11]. Pneumatic mixing differs operationally in that liquid circulation is generated within a stationary standard microplate, which may be advantageous for early SCC workflows requiring compatibility with conventional plate handling, imaging, and clone-tracking procedures. Direct comparison with alternative microplate-agitation approaches will be required to determine which benefits are specific to the pneumatic mechanism.

The workflow studies suggest that improved early expansion translated into faster downstream progression. In the single paired SCC campaign evaluated here, clones expanded under pneumatic mixing reached sufficient cell density for direct transfer from 24-well culture to shake-flask adaptation, whereas static-derived clones required an additional 6-well expansion stage to increase viable cell density and cell number before suspension transfer. As a result, the pneumatic-mixing workflow progressed to suspension adaptation 3 days earlier and to the fed-batch production stage 7 days earlier than the static workflow. Because these values derive from one independent SCC campaign, they should be interpreted as workflow-specific observations rather than statistically established effect sizes across independent CLD campaigns.

The additional 6-well stage in the static arm was therefore not an arbitrary experimental difference but a practical consequence of insufficient expansion after static 24-well culture. Nevertheless, because the two arms experienced different pre-adaptation histories, the downstream comparison remains a workflow-level comparison rather than an isolated test of mixing intensity. Notably, even after the static arm received this additional expansion period, the pneumatic-mixing workflow included four clones with final absolute titers and four clones with qP values above the respective maxima observed in the static workflow, showing that higher downstream performance was observed even after the static arm received an additional expansion period.

The clone-rank analysis further clarified the relationship between early screening and downstream performance. Among the eight clones advanced to fed-batch production, 96-well relative titer was positively correlated

with final fed-batch titer in the pneumatic-mixing workflow (Spearman's $\rho=0.7857$, $P=0.0279$), while the static workflow showed a weak negative, non-significant correlation ($\rho = -0.4286$, $P=0.2992$). At the 24-well stage, the pneumatic-mixing workflow showed a positive correlation that did not reach statistical significance ($\rho=0.7143$, $P=0.0576$), while the static workflow showed a weak negative, non-significant correlation ($\rho = -0.2143$, $P=0.6191$). These data were consistent with greater preservation of clone ranking under the tested pneumatic-mixing conditions; however, the workflow-specific correlation coefficients were not formally compared, and the analysis was restricted to an enriched subset because the clones entering fed-batch production had already been selected according to early relative titer. Therefore, the analysis does not establish predictive accuracy across the original unscreened clone population.

The integrated production workflow was demonstrated using a single anti-PD-L1-producing CHO model, and broader generalization will require validation across additional host backgrounds, recombinant molecules, media, and production conditions. In addition, although the 96- and 24-well pneumatic-mixing formats were used in separate experimental workflows in Figs. 4 and 5 and showed consistent qualitative behavior, a dedicated inter-plate or inter-chamber reproducibility study with predefined quantitative acceptance criteria was not performed. Such characterization will be important for future higher-throughput implementation.

Culture-process engineering represents one route to accelerating CLD, whereas complementary strategies act directly on host-cell growth and production phenotypes through metabolic, transcriptional, or genetic engineering [17–20]. These approaches are complementary rather than competing and may ultimately be combined to accelerate clone recovery, expansion, and downstream development.

Overall, the present study supports controlled pneumatic microplate mixing as a practical strategy to improve early CHO clone expansion and reduce the discontinuity between static clone outgrowth and later suspension culture. Its principal advantages in the tested workflow were improved early expansion and faster transition to suspension culture, while the observed correlation pattern was consistent with greater preservation of clone ranking among selected high-performing clones. Further validation across independent SCC campaigns, additional production models, and alternative agitation technologies is needed to define the generalizability and mechanism of these effects.

Conclusions

This study demonstrates that controlled pneumatic microplate mixing can improve the efficiency and continuity of early CHO clone development. Across platform characterization, benchmark comparisons, operating-window definition, clone-level validation, and an integrated SCC workflow demonstration, pneumatic mixing supported stronger early expansion, facilitated suspension adaptation, and enabled earlier progression to production-relevant stages. Among the selected clones advanced to production, the observed correlation pattern was consistent with greater preservation of clone ranking under the tested pneumatic-mixing conditions. Future studies across additional cell lines, molecules, media systems, and production modes will be needed to define the broader utility of the approach. Overall, pneumatic microplate mixing provides a practical strategy to improve early CHO clone expansion and reduce the discontinuity between static clone outgrowth and later suspension-based bioprocessing.

Abbreviations

ANOVA	Analysis of variance
CHO	Chinese hamster ovary
CLD	Cell line development
C.NEST	Pneumatic microplate mixing system
IVCC	Integral viable cell concentration
$k_L a$	Volumetric oxygen mass transfer coefficient

PBS	Phosphate-buffered saline
qP	Cell-specific productivity
SCC	Single-cell cloning
VCD	Viable cell density

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1186/s12896-026-01234-x>.

Acknowledgements We thank Yu-Kai Lai, Pheidias Che-Hao Wu, David Lenzen, and Chia-En Tsai for helpful discussions and comments on the manuscript.

Author contributions F.C.Y., Y.M.C., and S.P.L.: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, and Visualization. C.N.L.: Conceptualization, Methodology, Validation, Formal analysis, and Visualization. W.R.W.: Investigation. C.H.T.: Conceptualization, Methodology, Validation, Formal analysis, Writing - review & editing, Visualization, Supervision, and Project administration.

Funding This study received no external funding.

Data availability The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests Feng-Chun Yen, Yi-Ming Chiu, and Wei-Rou Wang are employees of Leadgene Biosolutions. Shih-Pei Lin was an employee of Leadgene Biosolutions during the conduct of this study, and Ching-Nan Lin was an employee of Leadgene Biomedical during the conduct of this study. Cheng-Han Tsai serves as Chief Executive Officer of Leadgene Biosolutions and is also a shareholder in Leadgene Biomedical. Leadgene Biosolutions was acquired by Leadgene Biomedical and formerly operated as CYTENA BPS. Leadgene Biosolutions previously had a corporate relationship with CYTENA GmbH; however, Leadgene Biosolutions and CYTENA GmbH are currently independent companies with no ownership relationship between them. The UP.SIGHT single-cell dispensing system and F.QUANT Fc Titer Assay used to generate data in this study are CYTENA products. Leadgene Biosolutions holds intellectual-property rights related to the C.NEST technology, including U.S. Patent No. 11,845,044 and pending U.S. Patent Application No. 18/038,397; European Patent EP 3 609 995 and pending European Patent Application EP 4 255 628; Taiwan Patent/Application TW 110 144 511; granted national equivalents of PCT/EP2018/059601, including in Australia, China, and India; and pending national equivalents of PCT/EP2021/083388 in China and Japan.

Open Access This article is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License, which permits any non-commercial use, sharing, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if you modified the licensed material. You do not have permission under this licence to share adapted material derived from this article or parts of it. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by-nc-nd/4.0/>.

References

1. Tihanyi B, Nyitray L. Recent advances in CHO cell line development for recombinant protein production. *Drug Discov Today Technol.* 2020;38:25–34.
2. Walsh G, Walsh E. Biopharmaceutical benchmarks 2022. *Nat Biotechnol.* 2022;40(12):1722–60.

3. Majumdar S, Desai R, Hans A, Dandekar P, Jain R. From Efficiency to Yield: Exploring Recent Advances in CHO Cell Line Development for Monoclonal Antibodies. *Mol Biotechnol*. 2024;67(2):369–92.
4. Plavsic M. ‘Q5D Derivation and Characterization of Cell Substrates Used for Production of Biotechnological/Biological Products’, in *ICH Quality Guidelines: an Implementation Guide*, A. E. D. N. R. W. Teasdale, Ed., Wiley, 2017, ch. 13, pp. 375–393.
5. Wurm FM, Wurm MJ. Cloning of CHO cells, productivity and genetic stability—a discussion. *Processes*. 2017;5(2):20.
6. Dorn M, Ferng C, Klottrup-Rees K, Lee K, Micheletti M. Cell clone selection—impact of operation modes and medium exchange strategies on clone ranking. *Front Bioeng Biotechnol*. 2024;12:1479633.
7. Neuss A, Steimann T, Tomas Borges JS, Dinger R, Magnus JB. Scale-up of CHO cell cultures: from 96-well-microtiter plates to stirred tank reactors across three orders of magnitude. *J Biol Eng*. 2025;19(1):5.
8. Wurm FM. CHO quasiespecies—implications for manufacturing processes. *Processes*. 2013;1(3):296–311.
9. Fisher JT, Gurney TO, Mason BM, Fisher JK, Kelly WJ. Mixing and oxygen transfer characteristics of a microplate bioreactor with surface-attached microposts. *Biotechnol J*. 2021;16(5):e2000257.
10. Hemmerich J, Noack S, Wiechert W, Oldiges M. Microbioreactor Systems for Accelerated Bioprocess Development. *Biotechnol J*. 2018;13(4):1700141.
11. Nienow AW, et al. The physical characterisation of a microscale parallel bioreactor platform with an industrial CHO cell line expressing an IgG4. *Biochem Eng J*. 2013;76:25–36.
12. Scherzinger J, Türk D, Aprile-Garcia F. An optimized and validated workflow for developing stable producer cell lines with >99.99% assurance of clonality and high clone recovery. *bioRxiv*. 2022:520697.
13. Aroniada M, Maina S, Koutinas A, Kookos IK. Estimation of volumetric mass transfer coefficient (kLa)—Review of classical approaches and contribution of a novel methodology. *Biochem Eng J*. 2020;155:107458.
14. Tsai C-H. Highly parallel microbioreactors for cell line development based on well plates with functional lids. University of Freiburg; 2019.
15. Fischer S, Handrick R, Otte K. The art of CHO cell engineering: A comprehensive retrospect and future perspectives. *Biotechnol Adv*. 2015;33(8):1878–96.
16. Kim JY, Kim YG, Lee GM. CHO cells in biotechnology for production of recombinant proteins: current state and further potential. *Appl Microbiol Biotechnol*. 2012;93(3):917–30.
17. Altamirano C, Cairó JJ, Gòdia F. Decoupling cell growth and product formation in Chinese hamster ovary cells through metabolic control. *Biotechnol Bioeng*. 2001;76(4):351–60.
18. Torres M, et al. Engineering mammalian cell growth dynamics for biomanufacturing. *Metab Eng*. 2024;82:89–99.
19. Marzluf J, Rattay M, Goertz M, Zehe C, Leroux AC. Genetic drivers of Chinese hamster ovary cell proliferation revealed by functional genomics. *iScience*. 2025;28(11).
20. Marzluf JP, Huckfeldt L, Haidas D, Zehe C, Leroux AC. A high-throughput co-culture CRISPR array for rapid fitness hypothesis testing and growth engineering in CHO cells. *iScience*. 2026;29(9):117201.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Authors and Affiliations

Feng-Chun Yen¹ · Yi-Ming Chiu¹ · Shih-Pei Lin¹ · Ching-Nan Lin² · Wei-Rou Wang¹ · Cheng-Han Tsai¹

✉ Cheng-Han Tsai
charles.tsai@leadgene-bio.com

¹ Leadgene Biosolutions, Taipei, Taiwan

² Leadgene Biomedical, Tainan, Taiwan