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S/C.NEST® | Cross-Contamination-Free Operation Validated in S/C.NEST Platform

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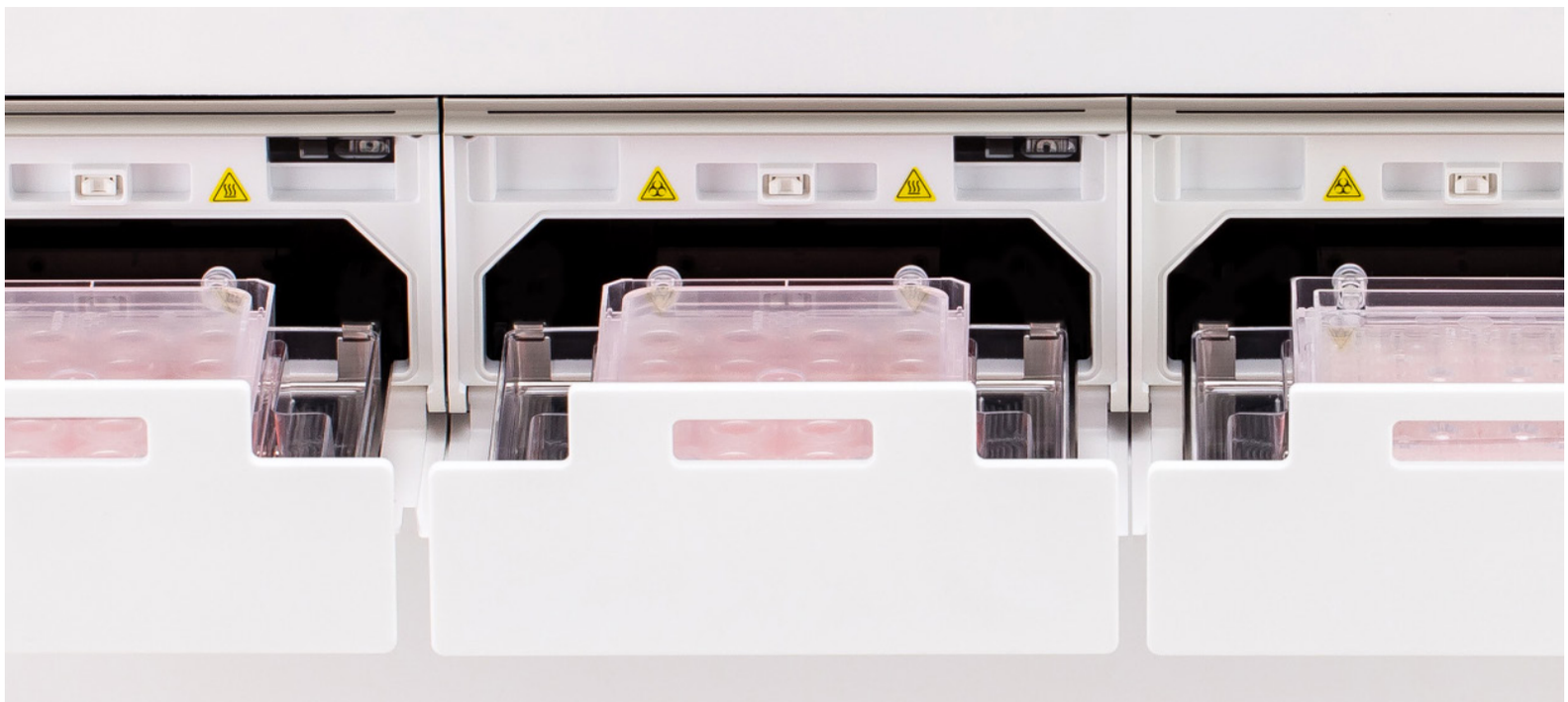
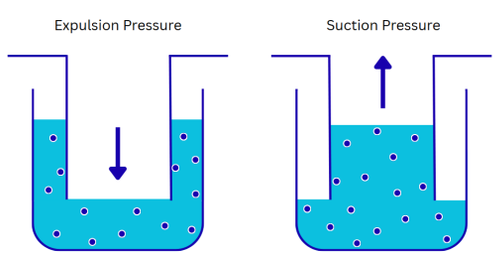


Introduction

In microscale bioprocessing, multi-well formats such as 24- and 96-well plates are essential for enabling high-throughput experimentation. The S/C.NEST microbioreactor developed by CYTENA BPS is designed to support these formats while minimizing the risk of cross-contamination. Using a patented air-driven mixing mechanism, S/C.NEST creates vertical fluid motion within each well through controlled airflow, delivered via a dedicated chamber and lid system. This airflow, sourced from both CO_2 cylinders and the surrounding atmosphere, is passed through a HEPA filter to maintain sterility—similar to standard incubators.



This mechanism helps to preserve the physical separation of each well, supporting a better

containment even under continuous or vigorous mixing. This method enables consistent, effective mixing while maintaining isolation between wells — a critical advantage for sensitive assays or experiments requiring high reagent purity. Still, validating the system's containment performance remains important, particularly in demanding applications.

This study was designed to assess well-to-well cross-contamination risk during active mixing in the S/C.NEST platform by utilizing a visible dye in a checkerboard pattern across a standard 24-well plate.

To detect even minimal cross-contamination, the experiment uses a series of diluted Trypan Blue standards. These standards provide a reference to quantify dye presence in wells and surrounding areas through optical density measurements, allowing sensitive detection of any unintended dye transfer during mixing.

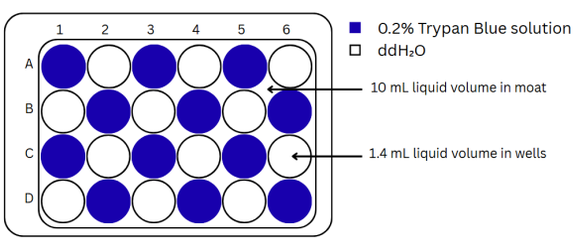
Materials and methods

A. Plate Setup and Reagents

A standard 24-well tissue culture plate was prepared using a checkerboard pattern in which alternating wells were filled with either:

- 1,400 µL of double-distilled water (ddH_2O), or
- 1,400 µL of a 0.2% Trypan Blue solution (prepared by diluting 0.4% stock 1:1 in ddH_2O).

This layout ensured that every Trypan Blue-filled well was completely surrounded by water-only "test wells". In addition to assessing well-to-well contamination, a separate focus of this experiment was to evaluate the potential for dye transfer between the moat (space between the wells) and the wells.



B. Device Configuration

The plate was incubated in a C.NEST equipped with an X.NEST Lid, and operated under the following conditions:

- Temperature: 37 °C
- CO_2 concentration: 5%
- Humidity: 90%
- Mixing Mode: Continuous with suspension boost
- Mixing Interval: Every 10 seconds

C. Experimental Conditions

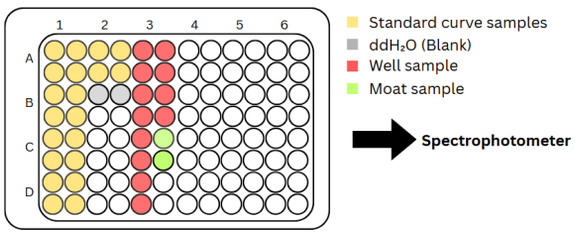
To assess the potential for cross-contamination, experiments were performed under two different operational conditions:

1. Frequent lid openings — to simulate real-world sampling with:
    - Manual lid handling by an experienced user
    - EZHold-assisted lifting to reduce lid contact and droplet formation.
2. Long-term culture — to evaluate whether the platform's in-well mixing action alone causes cross-contamination over extended periods.

EZHold is a mechanical lid support tool that features a knob-operated lift mechanism that minimizes direct contact between the lid and the microwell plate surface. This reduces the chance of droplet formation or lid-edge residue that may result in cross-contamination, especially during repeated sampling or component additions.

In this experiment, lid handling was performed by an experienced S/C.NEST user. While the results presented here reflect a controlled scenario with low risk of contamination, it is important to note that manual operation by less experienced users may introduce higher variability.

To simulate real-world sampling conditions, the X.NEST lid was repeatedly opened and closed at defined intervals over a 3-hour culture period. At each sampling point, 200 µL was withdrawn from every well and transferred to a 96-well plate for measurement of optical density at 595 nm (OD_{595}) using a spectrophotometer. Trypan Blue served as the visual tracer for potential cross-contamination, and a standard curve was prepared in advance to quantify even low-level dye transfer.



D. Sensitivity and Detection Threshold

To accurately quantify potential cross-contamination, a series of Trypan Blue standards with known dilutions was prepared prior to the experiment. Optical density at 595 nm (OD_{595}) was measured for each standard. This curve served as a reference to estimate dye concentrations in samples taken from test wells and the surrounding moat during the experiment.

To ensure accurate OD measurements, each well was sampled twice—200 µL was withdrawn twice as two separate replicates. Additionally, each 96-well plate was scanned three times to confirm consistency across readings and reduce variability.

By comparing OD_{595} readings of test samples to the standard curve, we could detect and quantify minimal amounts of dye — as little as a fraction of a microliter.

Results and discussion

Serial Dilution Curve

A standard dilution series of Trypan Blue was established before each experiment to correlate OD_{595} values with known dye concentrations. At each dilution, OD_{595} was measured for both the dye and a ddH_2O control blank. The blank's OD_{595} value was subtracted from the known dilution's OD_{595} values to obtain the corrected dye absorbance.

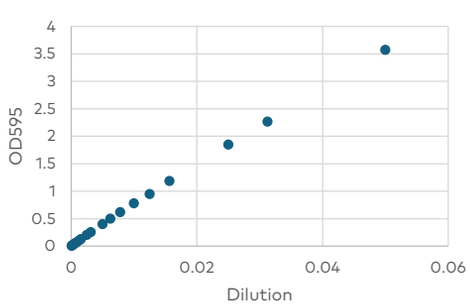


Figure 1 : Trypan blue dilution curve

Although multiple dilution ratios were evaluated, this paper highlights the 100,000×, 10,000×, and 1,000× dilutions for clarity and relevance.

The expected amount of 0.4% Trypan Blue in each case is:

- At 100,000× dilution, up to 0.014 µL of 0.4% Trypan Blue may be present in the solution.
- At 10,000× dilution, up to 0.14 µL is present.
- At 1,000× dilution, up to 1.4 µL is present.

To put this into perspective, a single water droplet from a pipette tip or condensation typically measures around 1–2 µL. This volume is equal to or greater than the dye content in the 1,000× dilution, and substantially higher than in 10,000× or 100,000×. Therefore, even a minor cross-contamination event — such as the transfer of a single droplet of 0.2% Trypan Blue — would be detectable using this dilution curve. This makes it a sensitive and reliable reference for contamination monitoring.

Frequent Lid Openings – Manual Handling

The experiment began with 2 hours of continuous mixing, followed by a sequence of lid openings with 15-minute mixing intervals. In total, four lid openings were performed: the first after 2 hours, and the remaining three at 15-minute intervals after.

After each lid opening, samples were transferred from the test wells into a 96-well plate for OD_{595} measurement. The OD value of the ddH_2O blank (control) was subtracted from each measurement to yield a corrected OD value. The results obtained after the fourth lid opening is shown below:

OD value

	0.037		0.040		0.037
0.039		0.038		0.038	
	0.037		0.036		0.035
0.036		0.036		0.035	
				Moat:	0.037

(OD value) - (blank OD value)

	0.001		0.004		0.001
0.003		0.002		0.002	
	0.001		0		-0.001
0		0		-0.001	
				Moat:	0.001

Table 1 : Test wells corrected OD_{595} value - Frequent opening using manual handling

Across all time points and lid openings, corrected OD_{595} values in the ddH_2O test wells remained low—ranging between 0.000 and 0.006—with no progressive increase observed over time.

These results were compared with the Trypan Blue standard dilution curve:

- At 100,000x dilution, the OD_{595} was 0.000.
- At 10,000x dilution, the OD_{595} was 0.007.
- At 1,000x dilution, the OD_{595} was 0.0825.

Since the test well readings remained below 0.006, any potential dye presence was below the detection limit defined by the 10,000x dilution, confirming the absence of meaningful cross-contamination.

Frequent Lid Openings – EZHold Handling

To assess whether EZHold impacts the risk of cross-contamination during frequent lid openings, a parallel test was conducted using the same dye-based approach. The experiment began with 1 hour of continuous mixing, followed by four sequential lid openings, each spaced by a 15-minute mixing interval.

OD value

	0.037		0.036		0.036
0.036		0.036		0.036	
	0.036		0.036		0.036
0.036		0.036		0.036	
				Moat:	0.036

(OD value) - (blank OD value)

	0.001		0.000		0.000
0.000		0.000		0.000	
	0.000		0.000		0.000
0.000		0.000		0.000	
				Moat:	0.000

Table 2 : Test wells corrected OD_{595} value - Frequent opening using EZHold

OD_{595} measurements of the ddH_2O test wells remained consistently low throughout the experiment, ranging between 0.000 and 0.001, indicating no measurable dye transfer.

Similar with the manual handling test, comparisons were made with the Trypan Blue dilution curve to evaluate detection sensitivity:

- At 100,000x dilution, the OD_{595} was -0.0005.
- At 10,000x dilution, the OD_{595} was 0.007.
- At 1,000x dilution, the OD_{595} was 0.0825.

Since the test well readings never exceeded 0.001, the dye concentration remained well below the detection threshold defined by the 1:20,000 dilution.

Long-Term Culture – EZHold Handling

To simulate extended culture conditions, three separate plates were incubated with continuous in-well mixing for different durations: one for 3 days, one for 7 days, and one for 14 days. At the end of each respective culture period, the lid was opened, and the wells were assessed for potential dye transfer. In addition to the dye dilution curve, a static (no-mixing) control group was included to serve as a baseline for comparison.

OD value

	0.037		0.036		0.036
0.036		0.037		0.036	
	0.037		0.036		0.036
0.036		0.036		0.036	
				Moat:	0.036

(OD value) - (blank OD value)

	0.00125		0.000		0.000
0.001		0.002		0.001	
	0.001		0.001		0.001
0.001		0.000		0.001	
				Moat	0.001

Table 3 : Test wells corrected OD_{595} value - Long-term static culture

OD value

	0.036		0.036		0.037
0.037		0.036		0.036	
	0.035		0.035		0.035
0.037		0.035		0.036	
				Moat:	0.036

value) -

	0.0001		0.001		0.002
0.001		0.001		0.001	
	0.000		0.000		0.000
0.001		0.000		0.001	
				Moat:	0.001

Table 4 : Test wells corrected OD_{595} value - Long-term mixing culture

OD_{595} measurements from the ddH_2O test wells across all plates—regardless of duration—remained low, ranging from 0.000 to 0.002, suggesting no evidence of dye cross-contamination even after two full weeks of continuous mixing.

Detection sensitivity was again benchmarked against the Trypan Blue dilution curve:

- At 100,000x dilution, the OD_{595} was -0.0005.
- At 10,000x dilution, the OD_{595} was 0.0085.

- At 1,000x dilution, the OD_{595} was 0.096.

Even under long-term mixing conditions and with periodic lid opening, OD values in the test wells remained far below the lowest detectable levels on the curve.

Conclusion

This study evaluated whether cross-contamination occurs in the C/S.NEST platform under typical and laboratory conditions, using a Trypan Blue dilution curve as a sensitive detection reference. The dilution curve confirmed that even extremely small volumes of dye—equivalent to a single droplet—can be reliably detected using OD_{595} measurements, providing a sensitive benchmark for monitoring contamination.

Throughout all tests, including manual and EZHold-assisted lid openings as well as long-term culture up to 14 days with continuous mixing, OD_{595} values from ddH_2O test wells remained consistently below the detection threshold. No accumulation over time was observed, and values stayed lower than those associated with the 10,000× dilution (0.14 µL dye in 1400 µL), indicating no meaningful dye transfer.

Importantly, all OD_{595} measurements taken from the moat—the space between wells—also remained at or near zero, further confirming that no cross-contamination occurred from the moat into neighboring culture wells when the platform was handled carefully.

Together, these results validate that the C/S.NEST platform maintains well isolation and spatial separation, even during frequent lid access and extended use. This makes it a robust option for contamination-sensitive applications in cell culture and beyond.

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