Efficient and consistent dissociation of adherent cell cultures using TrypsiNNex®

Performance assessment and strategy for using the new recombinant trypsin TrypsiNNex® in passaging HEK293, MDCK, MRC5, BM-MSC, UC-MSC, and AD-MSC cell lines.

Consistency is an essential aspect of biopharmaceutical manufacturing that is impacted by the quality and performance of the raw materials used. Also the uniformity of enzymes, like trypsin, that are used to manage adherent cell cultures contribute to achieving a consistent product quality. In this study, we assess the performance and efficiency of TrypsiNNex® in cell dissociation and profile its activity and compositional stability over time. Our results demonstrate that TrypsiNNex® is an equivalent alternative to commonly used commercial trypsin products, displaying high enzymatic activity and compositional purity over a 2-week period. TrypsiNNex® efficiently passaged viable cells, establishing its suitability for use in biopharmaceutical processes.

Trypsin quality is essential in biopharmaceutical production

Robust biopharmaceutical production is ensured by robust raw materials. In the case of manufacturing processes that rely on adherent living cells as producers, trypsin is a particularly influential input. Used optimally, trypsin efficiently detaches cells from their growth support during passaging, allowing continued population expansion. Used in excess, however, trypsin cleaves surface proteins and can curtail cell viability and replication.[1]

Whether used in virus propagation for vaccines[2], therapeutic protein expression, or vector manufacturing for cell and gene therapies[3], the reliable performance of trypsin is pivotal to facilitating rapid and reproducible cell population growth. Trypsin used in the manufacturing of medicinal products must exhibit predictable activity throughout a reasonable shelf life while being free of any agents that can potentially contaminate or infect the producer cells.

This study examines the performance of TrypsiNNex®, a new recombinant trypsin, in the dissociation of adherent cells. We first describe the key features underlying its enzymatic activity and safety profile. Then, we test the stability of its composition and activity over time and through freeze-thaw cycles. Finally, we compare the efficiency of TrypsiNNex® to other commercially available trypsin products in passaging different cell lines commonly used in biopharmaceutical manufacturing.

TrypsiNNex®: Delivering reliability and consistency through purity

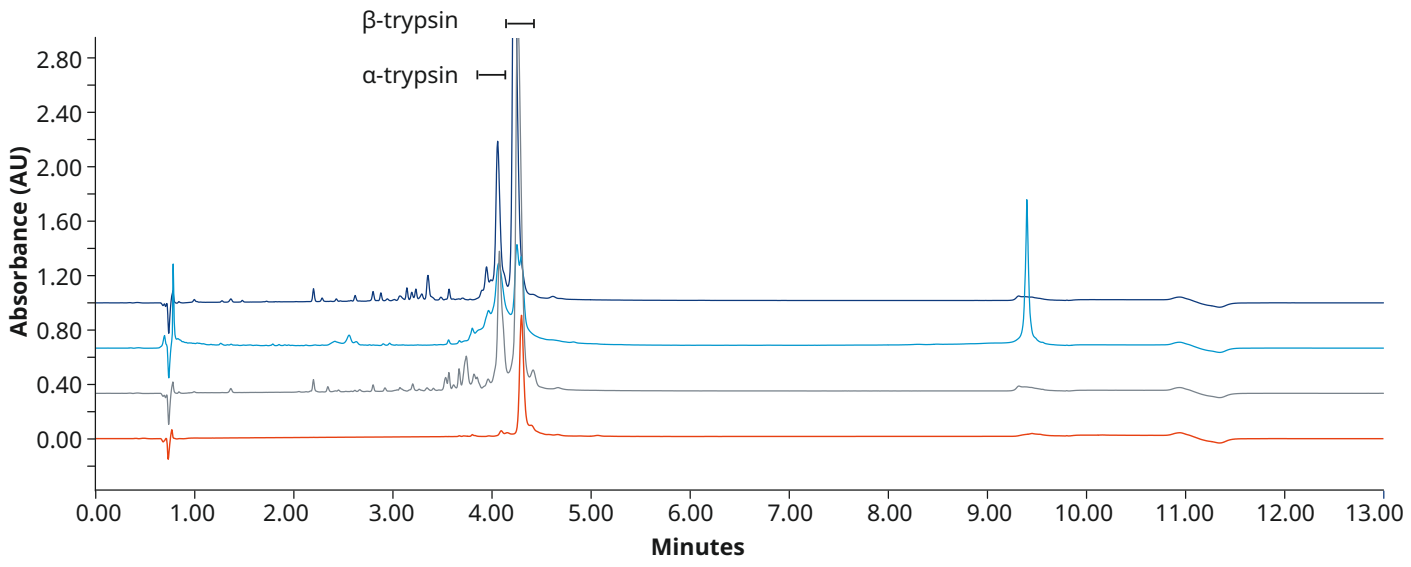
TrypsiNNex® is designed to perform consistently and, thus, close a significant gap in the narrow performance tolerances for biomanufacturing raw materials. That consistency is achieved by enforcing two aspects of purity. The first aspect is the animal component-free production of TrypsiNNex®. Viruses and other adventitious agents that have caused costly contamination events in processes using animal-derived trypsin are averted through recombinant production and stringent quality control. This means that TrypsiNNex® can be integrated into new and existing manufacturing processes to eliminate safety risks (see highlight box below "*The risk of viruses in raw materials*").

The second aspect is the high purity and consistent proteolytic activity of each TrypsiNNex® lot. Variation in enzymatic activity is regularly observed within and among lots of commercial trypsin products. A common culprit of those deviations is autolysis. Trypsin is an unstable molecule and self-degrades, producing decomposition fragments with reduced activity and altered specificity.[5] As a result, trypsin products are a mixture of fully active enzyme (β-trypsin), subpar enzymes (e.g., α-trypsin), and other components. This autodegradation can downgrade the overall performance of a mixture and lead to differences among enzyme lots depending on reaction conditions.[6]

To control the composition of TrypsiNNex® lots, autolysis during production is restricted. TrypsiNNex® is expressed in *Escherichia coli* cultures as an inactive protein subsequently activated during purification. With stabilization shortly thereafter, premature degradation is averted, and a substantial proportion of intact trypsin (β-trypsin) is safeguarded. In fact, the overall purity of TrypsiNNex® measured via reversed-phase high-performance liquid chromatography (RP-HPLC) exceeds 90%. The amount of β-trypsin in TrypsiNNex® is at a minimum 20 percentage points higher than in other tested commercial trypsin products relative to total HPLC absorbance at 215 nm (Figure 1).

The risk of viruses in raw materials

Trypsin is often sourced from porcine or bovine pancreatic tissue. Though typically less expensive, animal-derived trypsin carries the risk of harboring viruses and other adventitious agents that can adversely affect cell lines or the safety of a final product. The consequences of an infected cell line can be millions of dollars in production downtime, extensive corrective action, limited supply of much-needed medicines, and the danger of infecting patients. Furthermore, an industry-wide study of viral contamination risk in manufacturing processes highlighted that testing alone is insufficient to guarantee a product free of introduced viruses.[4]



	α-trypsin (%)	β-trypsin (%)	Total (%)
Product 1	13.9	67.7	81.6
Product 2	15.1	66.7	81.8
Product 3	19.0	65.4	84.4
TrypsiNNex®	5.3	89.3	94.6

Figure 1. Purity analysis of TrypsiNNex® and 3 other commercial trypsin products. Analysis by reversed-phase high-performance liquid chromatography (RP-HPLC) shows that TrypsiNNex® (red) has higher purity than alternative products, with a higher overall trypsin content and a greater proportion of intact trypsin (β-trypsin).

Robust performance over time and repeated freeze-thaw cycles

TrypsiNNex® should be diluted before use. While the shelf life of the stock solution is 2 years when stored at –20°C, the stability of a working solution is an important consideration in process development. Changes in the composition and activity of TrypsiNNex® working solutions stored at 4°C (Figure 2) and 21°C (data not shown) were tested over time. Two working solutions were created by diluting TrypsiNNex® in phosphate buffer saline (PBS) with 1 mM EDTA to 70 U/mL and 700 U/mL. The working solutions were stored at the test temperatures and sampled at different time points for up to 6 months. Total trypsin and the proportion of β-trypsin versus α-trypsin were profiled via reversed-phase high-performance liquid chromatography (RP-HPLC). The proteolytic activity of the TrypsiNNex® working solutions was measured as the change in absorbance per unit time (defined in USP <89>) resulting from the hydrolysis of the Chromozym TRY substrate (CustomBiotech, Germany).

Although the total trypsin measured remained relatively constant over time, TrypsiNNex® degraded in PBS with EDTA. The ratio of β-trypsin to α-trypsin declined

as intact trypsin autolyzed into increasing amounts of α-trypsin. The rate of autolytic decomposition, however, was decelerated at the lower enzyme concentration and storage temperature. Stored at 4°C, the 70 U/mL working solution retained more intact β-trypsin than α-trypsin for more than 6 months. Importantly, the initial ratio was stable for approximately 30 days (Figure 2A) before showing the first signs of degradation. In the 700 U/mL working solution, degradation occurred from the beginning and the ratio flipped in favor of α-trypsin after roughly 30 days (Figure 2C). Temperature had a strong effect on compositional stability, with both working solutions exhibiting a predominance of subpar α-trypsin within a few days after storage at 21°C (data not shown).

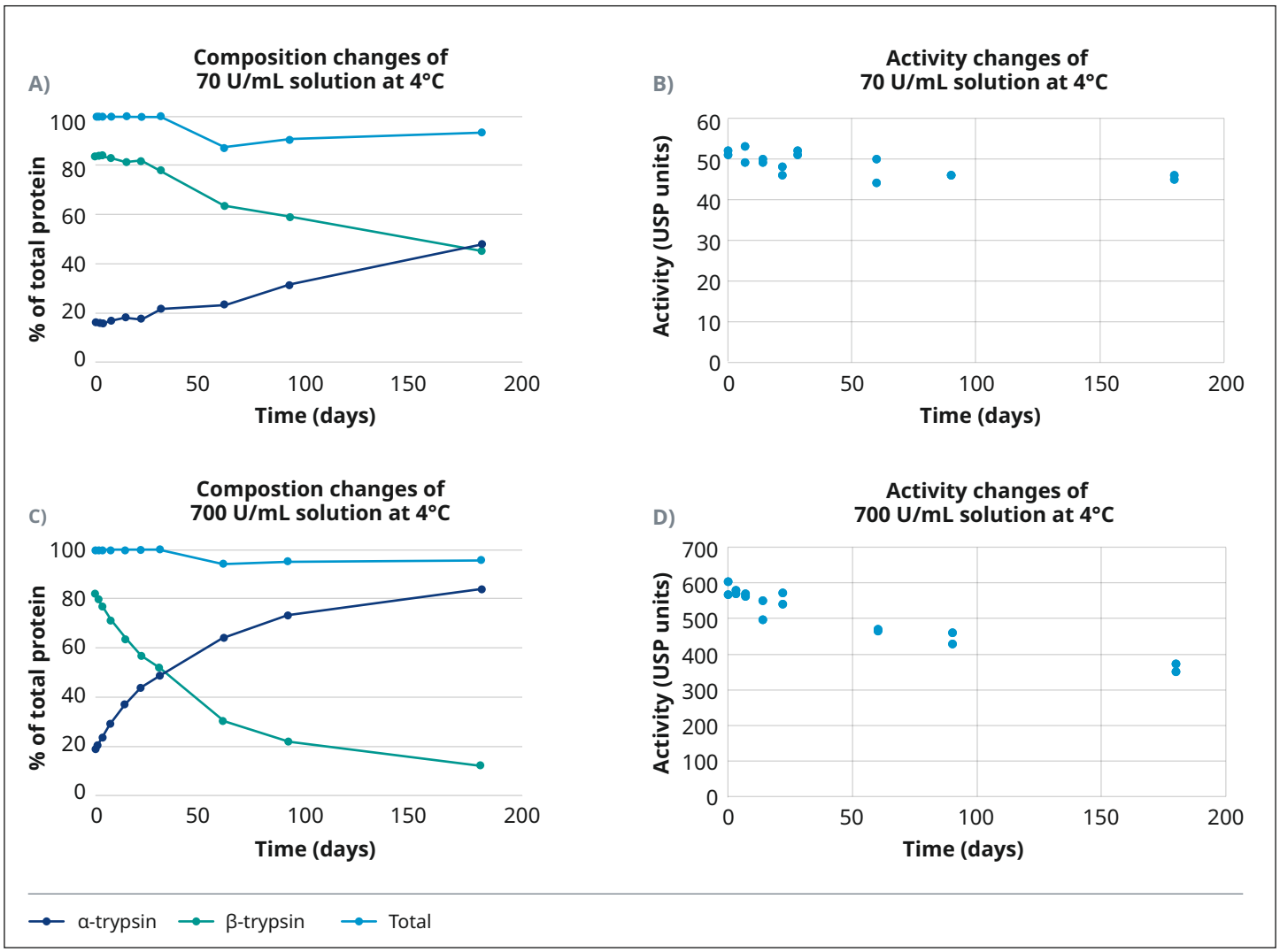


Figure 2. Changes in composition and activity of TrypsiNNex® working solutions stored at 4°C for 6 months. RP-HPLC analysis of TrypsinNNex® working solutions at 2 different concentrations and stored at 4°C showed different compositional dynamics. Depicted is the percent of the total protein corresponding to α-trypsin and β-trypsin. Both the 70 U/mL (A) and 700 U/mL (C) solutions had stable and high total trypsin amounts (turquoise line) during the 6 months of storage. Autolytic conversion of β-trypsin (green line) to α-trypsin (dark blue line) occured later for the 70 U/mL solution than for the 700 U/mL solution. Proteolytic activity was measured as the absorbance change per unit time following the hydrolysis of Chromozym TRY substrate (CustomBiotech, Germany). The results, reported in USP units, showed a slower activity decline in the 70 U/mL working solution (B) compared to its more concentrated counterpart (D).

The observed shift from β-trypsin to α-trypsin was accompanied by an expected decay in proteolytic activity (Figure 2B, 2D). With 38% activity loss over 6 months, the decline was more pronounced in the 700 U/mL working solution. The less concentrated working solution exhibited only a 12% decline in the same timeframe (Figure 2B). Based on these results, we recommend storing working solutions of TrypsiNNex® at 4°C and using them within 14 days where composition remains steady and activity remains high. A 2-week window where a TrypsiNNex® working solution retains consistently high β-trypsin and proteolytic activity provides flexibility in manufacturing processes.

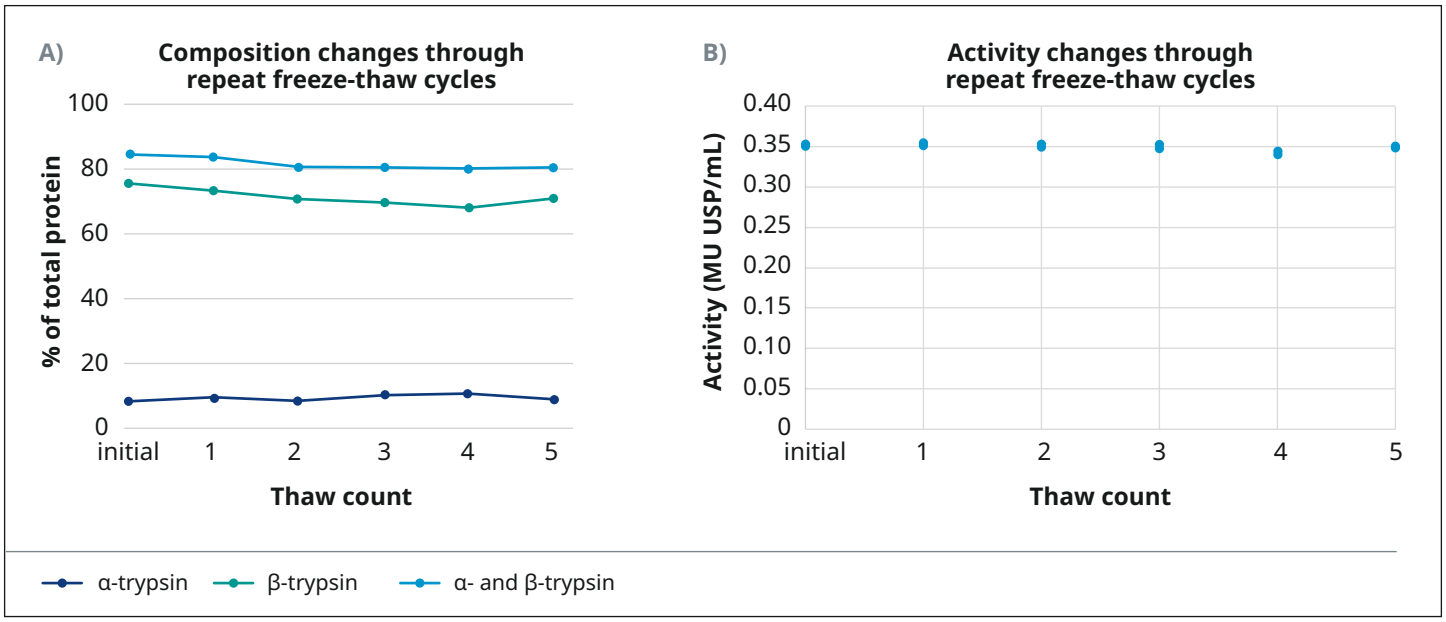


Figure 3. Composition and activity of a TrypsiNNex® stock solution over 5 freeze-thaw cycles. Repeated thawing and freezing (5 cycles) of a TrypsiNNex® stock solution did not adversely affect its composition or activity. RP-HPLC analysis showed that β-trypsin concentration remained high (A), demonstrating that freezing at –20°C prevented autolysis. Proteolytic activity (B) measured spectrophotometrically in duplicate samples using Chromozyme TRY substrate (CustomBiotech, Germany) was consistent from cycle to cycle.

We also assessed the effect of repeated thawing on the quality of TrypsiNNex®. We thawed a stock solution in an ice bath, collected a sample for RP-HPLC analysis and activity assessment, and then refroze the stock solution at –20°C. We repeated this freeze-thaw cycle 5 times. Figure 3 summarizes the results of all thaw events. TrypsiNNex® had consistently ≥80% total trypsin, with β-trypsin comprising >70% and α -trypsin about 10% of the stock solution. Proteolytic activity was also rigorously consistent across all freeze-thaw cycles. TrypsiNNex® stock solution can be safely thawed and refrozen repeatedly to create working solutions as needed.

We recommend storing TrypsiNNex® working solutions at 4°C. With a concentration of 70 USP/mL, the proportion of β-trypsin exceeds α-trypsin and proteolytic activity remains stable for 6 months. At 700 USP/mL, that stability is retained for 1 month. TrypsiNNex® stock solutions tolerate at least 5 freeze-thaw cycles without compositional changes or activity loss.

TrypsiNNex® effectively dissociates different cell lines

The efficacy of TrypsiNNex® as a dissociation reagent was tested on 6 cell lines: human embryonic kidney cells (HEK293), Madin-Darby canine kidney cells (MDCK), human fetal lung fibroblast cells (Medicinal Research Council strain 5, MRC5), bone marrow-derived mesenchymal cells (BM-MSC), umbilical cord-derived mesenchymal cells (UC-MSC), and adipose-derived mesenchymal cells (AD-MSC). Cells were seeded into 12-well plates or PET-based macrocarriers for 2-dimensional or 3-dimensional culture, respectively. After reaching 90% confluence, the cells were rinsed with PBS and incubated with different trypsin products. Depending on cell line, different concentrations and incubation times were tested. Trypsinization was stopped with culture medium and dissociated cells were counted and assessed for viability using a NucleoCounter® NC-200™ (Chemometec, Denmark). All experiments were conducted by an external laboratory.

TrypsiNNex® performed at the same level as other commercial trypsin products in detaching cells from culture surfaces while maintaining high cell viability. Table 1 summarizes the results of the experiments, highlighting the concentration and incubation time that maximized viable cell density and percent viable cells after trypsinization with each tested trypsin product.

A description of the experimental methods and more detailed results are provided in the supplementary material "*Efficient and consistent dissociation of adherent cell cultures using TrypsiNNex®*". Download the document from the TrypsiNNex® application page at novonordiskpharmatech.com

Table 1. Conditions for efficient cell dissociation using different trypsin products and cell lines

		HEK293	MDCK	MRC5		BM-MCS		UC-MSC		AD-MCS	
Enzyme		**2D**	**2D**	**2D**	**3D**	**2D**	**3D**	**2D**	**3D**	**2D**	**3D**
TrypsiNNex®	Concentration (U/mL)	200	1000	300	300	30	300	30	300	300	30
	Incubation (min)	5	10	4	30	2	30	2	30	2	30
	Cell density (cells/mL)	1.57E+5	3.9E+05	1.53E+5	2.39E+5	1.8E+5	2.37E+5	1.82E+5	3.29E+5	1.94E+5	3.18E+5
	Cell viability (%)	96.1	100	97.2	97.8	98.4	98.3	96.3	98.3	98.1	98.1
	+ EDTA (mM)	–	–	0.5	–	–	–	0.5	–	0.5	1
Shellfish-derived reagent	Concentration	1X	1X	1X	1X	1X	1X	1X	1X	1X	1X
	Incubation (min)	10	20	4	30	2	30	2	30	2	30
	Cell density (cells/mL)	1.6E+5	3.14E+5	1.42E+5	2.97E+5	2.15E+5	2.27E+5	1.49E+5	2.61E+5	1.89E+5	3.03E+5
	Cell viability (%)	98.2	99.6	95.6	94.7	98.1	96.5	95.7	95.9	99.1	95.2
Recombinant fungal trypsin-like enzyme	Concentration	1X	1X	1X	1X	1X	1X	1X	1X	1X	1X
	Incubation (min)	5	20	4	30	2	30	2	30	2	30
	Cell density (cells/mL)	1.64E+5	3.27E+5	1.34E+5	2.04E+5	2.27E+5	2.77E+5	1.52E+5	2.30E+5	1.71E+5	2.99E+5
	Cell viability (%)	96.1	99.8	94.5	95.8	98.2	98.1	95.5	97.0	98.6	98.8
Porcine-derived trypsin	Concentration (%)	0.25	0.25	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
	Incubation (min)	5	20	4	30	2	30	2	30	2	30
	Cell density (cells/mL)	1.68E+5	2.86E+5	1.21E+5	2.02E+5	1.63E+5	1.98E+5	1.29E+5	189E+5	1.79E+5	2.34E+5
	Cell viability (%)	96.1	99.1	94.5	95.8	96.3	97.5	95.4	98.3	98.7	98.9

In summary, TrypsiNNex® at 200 U/mL efficiently dissociated HEK293 cells and achieved over 90% cell viability after dissociation for nearly all tested incubation times (see supplementary document). As with the benchmark trypsin products, a shorter incubation tended to lower cell viability and increase variation in viable cell density. Extending incubation to 5 or 10 minutes allowed for maximum cell dissociation without sacrificing viability. TrypsiNNex® effectively detached HEK293 cells with a 5-minute incubation, performing on par with the other commercial trypsin products.

A 10 and 20-minute incubation period allowed efficient dissociation of MDCK cells by all tested trypsin products, including TrypsiNNex®. Cell viability was uniformly above 90%, and post-dissociation monitoring showed good cell recovery (data not shown). The 5-minute incubation period produced results that were often below the detection limit of the cell counter used. Therefore, measurements of cell viability were not accurate in those cases. Finally, TrypsiNNex® was also highly effective in dissociating 2-dimensional cultures of MSCs and MCR5 cells, performing at the same level as other commercial trypsin products.

TrypsiNNex® concentrations of 30 and 300 U/mL were highly effective at detaching cells from PET-based macrocarriers for all 3-dimensional (3D) cell cultures tested – mesenchymal stem cells (BM-MSC, AD-MSC, UC-MSC) and MRC5 cells (see supplementary document). At these concentrations, TrypsiNNex® performed well within the range of commonly used commercial trypsin products.

In some instances, the addition of EDTA to TrypsiNNex® improved results, though changes were not substantial. However, post-harvest monitoring showed that high concentrations of EDTA decreased cell viability, especially in MRC5 cells (data not shown). We advise diluting TrypsiNNex® in PBS with a low concentration of EDTA when dissociating MRC5 cells.

We recommend the following incubation times and TrypsiNNex® concentrations to passage 2D cultures of cell lines tested in this study:

- 2 minutes in 30 U/mL TrypsiNNex® for BM-MSC and UC-MSC
- 2 minutes in 300 U/mL TrypsiNNex® for AD-MSC
- 4 minutes in 300 U/mL TrypsiNNex® for MCR5 cells
- 5 minutes in 200 U/mL TrypsiNNex® for HEK293 cells
- 10 minutes in 1000 U/mL TrypsiNNex® for MDCK cells

The addition of low-concentration EDTA can improve results.

To passage 3D cell cultures, we recommend:

- 30 minutes in 300 U/mL TrypsiNNex® for BM-MSC, UC-MSC, and MCR5 cell lines
- 30 minutes in 30 U/mL TrypsiNNex® for AD-MSC

The addition of low-concentration EDTA can improve results.

Under the conditions listed above, the performance of TrypsiNNex® in dissociating cells is comparable to other commercial trypsin products.

Conclusions

Adherent cell culture systems are vital in biomanufacturing. As a key cell dissociation reagent that enables culture expansion, trypsin can impact the efficiency and scalability of a production process. The recombinant enzyme TrypsiNNex® was designed as a high-purity, animal component-free raw material that is easily integrated into existing and new protocols for cell culture passaging. This study demonstrated the stable composition and activity of TrypsiNNex®. Stored at 4°C, a 70 U/mL working solution of TrypsiNNex® retained a high proportion of β-trypsin and showed stable proteolytic activity for 2 weeks. In fact, β-trypsin exceeded α-trypsin in the solution for up to 6 months with some decline in activity due to the gradual autolysis. Additionally, a TrypsiNNex® stock solution tolerated at least 5 freeze-thaw cycles without changes in composition or performance. As a cell dissociation reagent, TrypsiNNex® proved to be equivalent to commonly used commercial trypsin products. These experiments provide guidelines for the use of TrypsiNNex® with different cell lines, outlining enzyme concentrations and incubation times that favor efficient passaging and high numbers of viable cells.

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