

# TrypLE™ Express: A Temperature Stable Replacement for Animal Trypsin in Cell Dissociation Applications

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## Abstract

TrypLE™ Express is a recombinant fungal trypsin-like protease, which has proven effective at dissociating many different attachment dependent mammalian cell lines. It has similar dissociation kinetics to porcine trypsin and exhibits lower cell toxicity. Cell replating, proliferation kinetics, and long-term maintenance were comparable to cells harvested using animal trypsin. HPLC analysis indicates a single peak corresponding to the active form of the recombinant enzyme, as opposed to the multiple peaks seen with animal (porcine pancreatic) trypsin.

Studies of TrypLE™ Express in solution have demonstrated remarkable enzyme stability. Enzyme assay results indicate that at 4°C and room temperature, the enzyme remains stable for more than 12 months. Even at 37°C, TrypLE™ Express maintains 85% activity for 8 days. In contrast, porcine trypsin lost greater than 95% of its trypsin activity after just 24 hours at 37°C. This temperature stability will allow more convenient storage and handling of this cell dissociation enzyme during routine cell culture procedures.

## Introduction

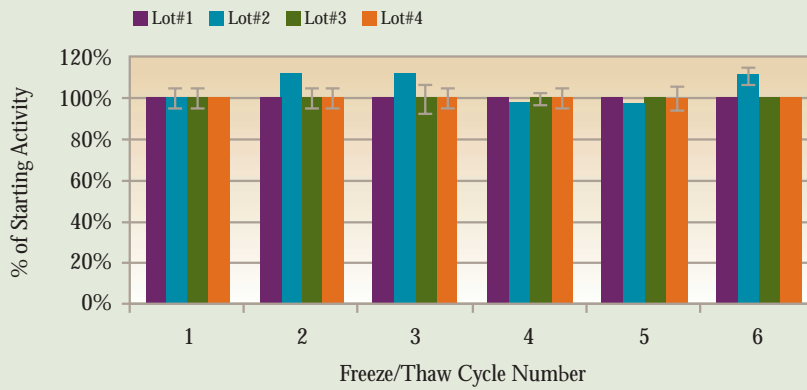
TrypLE™ Express is a high purity, recombinant fungal enzyme produced by fermentation. It is known to be a serine protease with trypsin-like activity (i.e. it cleaves at the same two amino acid sites—arginine and lysine—as trypsin and has a similar pH activity profile). This activity suggests that TrypLE™ Express will substitute for traditional porcine trypsin for dissociating cultured cells. It is available as a 1X solution prepared in phosphate buffered saline with 1mM EDTA, which was used throughout

these experiments. Porcine trypsin is unstable when stored unfrozen and suffers from relative instability at and above room temperature. Preliminary data suggests that TrypLE™ Express has superior stability compared to porcine trypsin. This study was designed to determine the purity and storage stability of TrypLE™ Express; the concentration at which it should be used; whether it needs to be inactivated with an inhibitor; if it is toxic to cells in the short or long term; and its ability to work on multiple cell lines.

## Materials and Methods

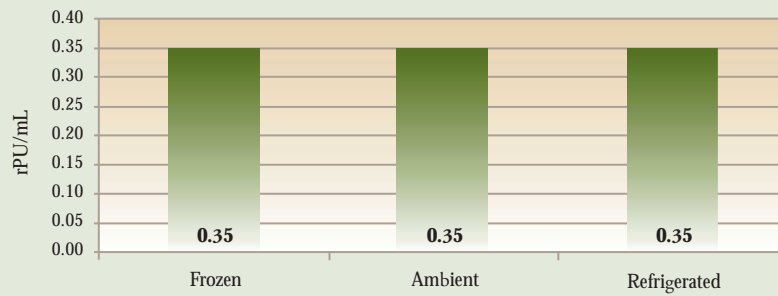
*Ac-Arg-pNA Protocol for Determining Tryptic Activity* (1): The Ac-Arg-pNA (acetyl arginine p-nitroaniline) enzyme assay was used to determine the activity of microbial trypsin-like enzymes. The liberated pNA produces an absorption increase at 405 nm, which is proportional to the enzyme activity. The TrypLE™ Express samples were formulated at a 1X working concentration in phosphate buffered saline with 1 mM EDTA. The results are given in rPU/mL.





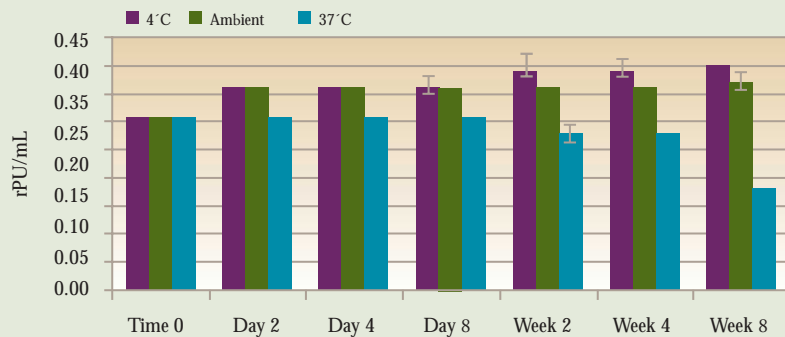
**Figure 1. Freeze/Thaw Stability.**

No loss in activity was seen on four different lots of TrypLE™ Express following 6 freeze/thaw cycles



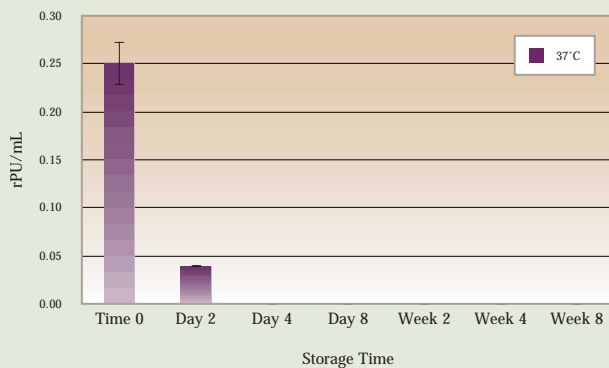
**Figure 2. 12-Month Shelf Life Data.**

Enzyme activity did not drop after 12 months of storage at frozen, ambient or refrigerated storage temperatures (in the dark).



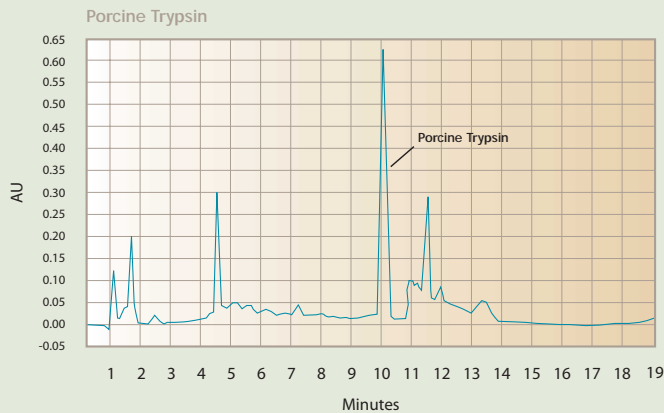
**Figure 3. TrypLE™ Express Stability.**

TrypLE™ Express shows superior stability compared to crude or purified porcine trypsin at various storage temperatures. Even after 8 weeks at 37°C, 50% enzyme activity was still seen. (All samples were stored in the dark.)



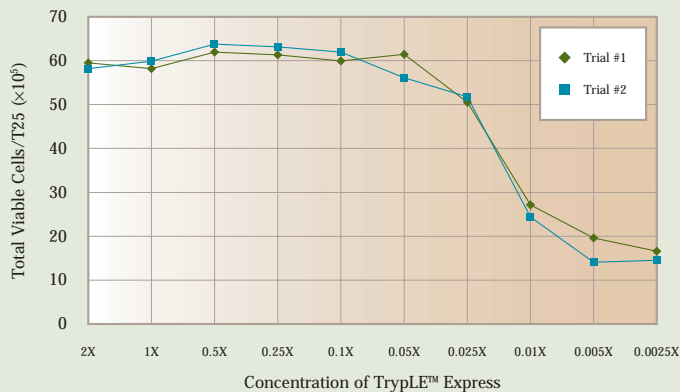
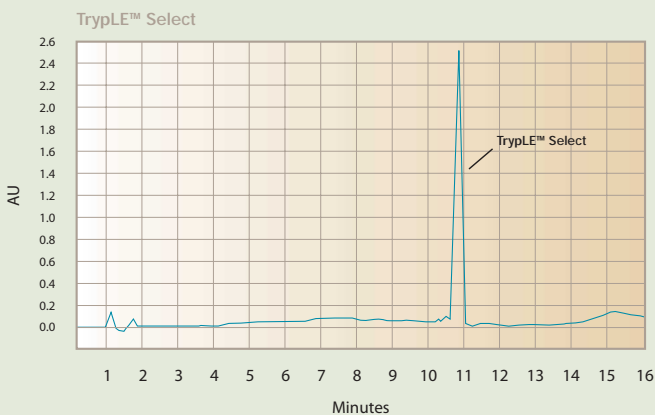
**Figure 4. Purified Porcine Trypsin Stability.**

Enzyme activity decreased rapidly in samples of purified porcine trypsin stored at 37°C.



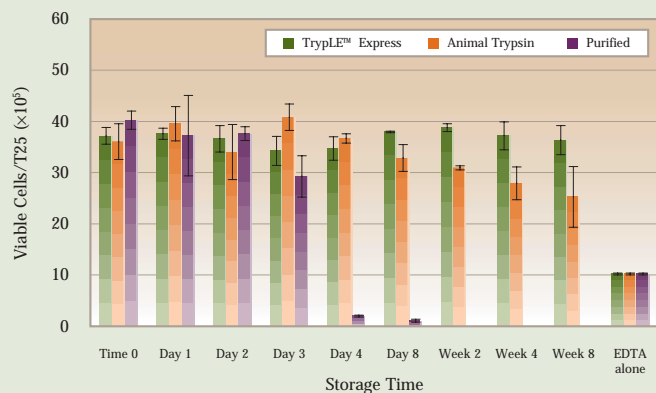
**Figure 5. Enzyme Purity.**

As seen in the HPLC chromatograph on the left, the multiple peaks in porcine trypsin indicate impurities. The chromatograph below left shows a single peak demonstrating the purity of TrypLE™ Express.



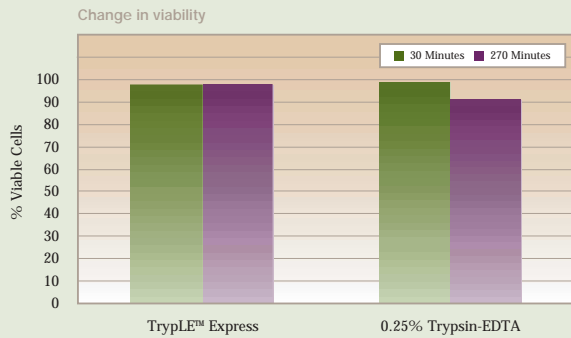
**Figure 6. Example of Concentration Response Curve.**

The half maximal concentrations for both trials shown in the figure were between 0.01X and 0.025X, which was well below the selected final working concentration of 1X.



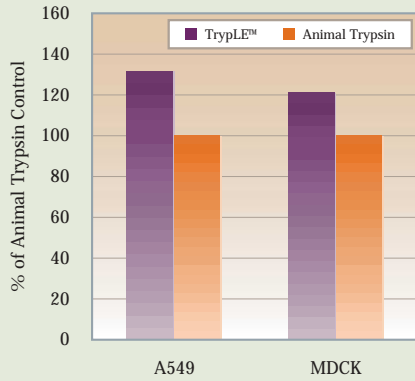
**Figure 7. Effect of 37°C storage on cell removal by crude porcine trypsin, purified porcine trypsin or TrypLE™ Express.**

The purified porcine trypsin failed to remove cells after 3 days mirroring the rapid loss in enzyme activity as seen in Figure 4. Crude trypsin starts to lose activity almost immediately, but still removes cells after 8 weeks of incubation possibly due to the action of the EDTA and contaminating proteases such as chymotrypsin and collagenase. Even after the 50% loss of enzyme activity (Figure 3), the 8 week TrypLE™ Express sample removed cells just as efficiently as the Time 0 sample.



**Figure 8. Short term toxicity on MDCK cells.**

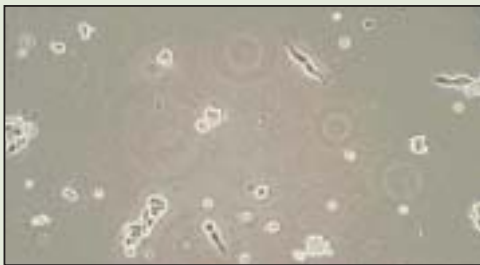
No loss in viability was observed after 270 minutes of exposure to TrypLE™ Express. Normal exposure time for MDCK cells is 30 minutes.



**Figure 9. Colony Plating Efficiency.**

For both cell lines tested, plating efficiency was >100% of the porcine trypsin control.

**Animal Trypsin**



**Figure 10. MDCK cell dissociation without washing or the use of trypsin inhibitors.**

Cells were not washed following dissociation and no protease inhibitors were used. Morphology was observed 24 hours later. While 1mM EDTA was needed for TrypLE™ Express to remove cells, protease inhibitors were never needed to preserve cell viability or plating efficiency. In fact, use of inhibitors reduced viability and growth rate at some concentrations.

**TrypLE™ Express**

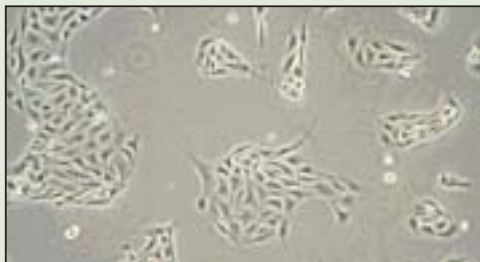


Table 1. Multiple Passage Assay TrypLE™ Express is effective on many common cell lines.

Cell Line Tested	Growth Medium	Mean Time Required for Cell Release	Mean Viability	Mean Cell Yield Expressed as Percent of Porcine Trypsin Control
VERO	VP-SFM	5 minutes	100%	104%
VERO	EMEM + 5% FBS	8 minutes	99.9%	98%
VERO	OptiPRO™ SFM	7 minutes	98%	130%
PK-15	EMEM + 5% FBS	27 minutes	98%	101%
PK-15	OptiPRO™ SFM	11 minutes	98.8%	106%
MDCK	OptiPRO™ SFM	28.5 minutes	98%	87%
MDBK	DMEM + 5% FBS	15 minutes	100%	94%
A549	DMEM + 5% FBS	9 minutes	98%	114%
293F	DMEM + 5% FBS	2 minutes	97.3%	131%
CHO-K1	CHO III A	7 minutes	95.3%	96%

As seen in the table, all cell lines tested met the acceptance criterion of greater than or equal to 80% of control. Cell viability remained >95% in all cell lines tested. Test samples were stored in the dark while not in use.

*rPU (Recombinant Protease Activity Unit):* One rPU is the quantity of enzyme that will convert 1.0 mmole of Ac-Arg-pNA substrate per minute at pH 8.0 and room temperature ( $22 \pm 1^\circ\text{C}$ ). Conversion factor: 1 rPU  $\approx$  293 USP trypsin units.

*HPLC Protocol:* The chromatographs shown in Figure 5 illustrate typical Hamilton PRP-3 column separations of trypsin samples. Mobile Phase A (0.1% Trifluoroacetic acid in HPLC grade water) and Mobile Phase B (0.1% Trifluoroacetic acid in HPLC grade acetonitrile) were used to wash and prepare the column for separation. A known trypsin standard was used to create a standard curve (correlation coefficient  $r^2 > 0.999$ ).

*Cell Lines:* The following cell lines were tested using TrypLE™ Express (both serum-free and serum-supplemented media were used): MDCK

(Madin Darby canine kidney), A549 (human lung carcinoma), VERO (African green monkey kidney), PK-15 (porcine kidney), MDBK (Madin Darby bovine kidney), 293F MSR (human embryonic kidney), and CHO-K1 (Chinese hamster ovary). These cell lines were tested for multiple passage toxicity and serial dilution dose response curves.

*Serial Dilution Protocol:* The optimal concentration of the final product was determined by serial dilution experiments. A series of 10 two-fold dilutions of TrypLE™ Express were tested on cells to determine the half maximal concentration. Cells were treated with dilutions for a fixed time, and dissociated cells were counted to determine total viable cells/mL. The half maximal (or 50% activity) concentration was determined from the dose response curve.

*Cell Removal with 37°C Samples—Fixed Treatment Time:* Aliquots of

TrypLE™ Express and purified trypsin were stored at 37°C (in the dark), and samples pulled at Time 0, Days 1, 2, 3, 4, and 8, and Weeks 2, 4, and 8. All samples contained 1 mM EDTA. MDCK cells were plated in T25 flasks at  $3 \times 10^5$  cells/flask in OptiPRO™ SFM. Three days later, cells were washed with DPBS, and 1 mL of the trypsin sample was added to each T25 flask. After 30 minutes, cells were washed with growth medium, and total viable cell counts were determined.

*Short Term Toxicity Protocol:* MDCK cells were treated with TrypLE™ Express and left on cells for 270 minutes. Viabilities were obtained at 30 and 270 minutes to test for possible toxicity. (Normal exposure time for MDCK cells is 30 minutes.)

*Multiple Passage Toxicity Protocol:* Cells were plated in T25 flasks at appropriate plating density and appropriate medium

for cell type. After the cells were washed with DPBS, 1 mL of TrypLE™ Express or porcine trypsin control was added to each T25 flask. After all cells dissociated from the plastic, cells were washed with growth medium and centrifuged at 1,000 rpm for 5 minutes. The cell pellet was resuspended in growth medium, and total viable cell counts were determined. The cells were seeded into new flasks, and the assay was repeated for six consecutive passages. The flasks were incubated at 37°C with 5% to 8% CO<sub>2</sub> (cell line dependent) and subcultured over a 3-day; 4-day passage cycle. Consecutive day 3 and day 4 passages initiated using a constant seed density were averaged, and an acceptance criterion of greater than or equal to 80% of control was used to assess performance of TrypLE™ Express.

**Plating Efficiency Protocol:** MDCK and A549 cells were used to test for colony plating efficiency. Cells were grown in appropriate growth medium and plated in 6-well plates at 100 and 200 cells/well. Colonies were stained with a 0.5% Methylene Blue solution and counted after 7 or 11 days (cell line dependent). Results were based on colony counts when compared to control wells.

**Trypsin Inhibitor:** MDCK cells were treated with traditional animal trypsin or TrypLE™ Express and replated into new T-flasks in OptiPRO™ SFM. Cells were not washed following dissociation, and no protease inhibitors were used.

## Results

**Ac-Arg-pNA Assay:** The pNA enzyme was used to determine the activity of microbial trypsin-like enzymes for the following:

- Freeze/Thaw Stability (Figure 1): No loss in activity was seen on 4 different lots following 6 freeze/thaw cycles.
- 12-Month Shelf Life Data (Figure 2): Enzyme activity did not drop after 12 months of storage at -20°C, 4°C, or ambient temperatures (all samples

stored in the dark).

- Comparison Stability (Figures 3 and 4): TrypLE™ Express showed superior stability to purified porcine trypsin stored at 37°C.

**Enzyme Purity (Figure 5):** As seen in the chromatograph, the multiple peaks indicate impurities. The TrypLE™ chromatograph shows a single peak demonstrating the purity of TrypLE™ Express.

**Serial Dilution Protocol (Figure 6):** The dose response curve shown in Figure 6 is a representative of all the cell lines tested. The half maximal concentration for both trials shown in the example was between 0.01X and 0.025X, which was well below the selected final working concentration of 1X. The 1X concentration is the concentration used in all experiments described. Similar results were found for all cell lines tested.

**Cell Removal of Samples Stored at 37°C (Figure 7):** The purified porcine trypsin failed to remove cells after 3 days mirroring the rapid loss in enzyme activity as seen in Figure 4. Even after the 50% loss of enzyme activity (Figure 3), the 8 week TrypLE™ sample removed cells just as efficiently as the Time 0 sample.

**Short Term Toxicity (Figure 8):** No loss in viability was observed after 270 minutes of exposure to TrypLE™ Express. Normal exposure time for MDCK cells is 30 minutes.

**Multiple Passage Assays (Table 1):** As seen in the table, all cell lines tested met the acceptance criterion of greater than or equal to 80% of control. Cell viability remained >95% in all cell lines tested.

**Colony Plating Efficiency (Figure 9):** For both cell lines tested, plating efficiency was >100% of control.


**No Need for Trypsin Inhibitor (Figure 10):** MDCK cells treated with traditional animal trypsin did not attach or spread out after 24 hours. Cells treated with TrypLE™ Express formed colonies and had good morphology after 24 hours.

## Discussion

The results of the various experiments presented here indicate that TrypLE™ Express performs equivalent to or better than animal trypsin and is therefore a suitable nontoxic, stable replacement for cell removal applications. Invitrogen's in-house shelf life testing program has demonstrated that TrypLE™ Express can be stored at 4°C or ambient temperatures (in the dark) for at least 12 months with no loss in activity or cell removal kinetics. 37°C incubation data indicates that TrypLE™ Express is extremely stable as compared to porcine trypsin, which makes it much more convenient for routine cell culture use.

TrypLE™ Express exhibits only 1 protein peak by HPLC, which represents the trypsin-like enzyme. The purified porcine trypsin activity and protein peak disappears after 1 week at 37°C, while TrypLE™ Express still has 100% enzyme activity. Only 50% enzyme activity is lost after 8 weeks at 37°C.

TrypLE™ Express is stable when subjected to repeat freezing and thawing. It has the same amino acid cleavage site as porcine trypsin and a similar pH profile activity (see reference to Novozyme's Patents; 2, 3). TrypLE™ Express maintains cell viability and plating efficiency, and it has been tested on multiple cell lines with comparable results to porcine trypsin.

In summary, TrypLE™ Express is superior to trypsin in purity, stability, and plating efficiency. Cell removal and proliferation kinetics are comparable to animal trypsin. 

## References

1. Internal QC Test: "Determining Activity for Microbial Trypsin-like Protease Using Ac-Arg-pNA."
2. Patent 5,288,627: Endoprotease from *Fusarium Oxysporum* DSM 2672 for Use in Detergents.
3. Patent 5,693,520: Recombinant Trypsin-like Protease.