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Enhancing sample throughput for deep dive proteomics with Tandem LC

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Abstract

Purpose: Explore the potential of a novel, intelligent tandem LC-MS workflow with near 100% MS utilization for deep dive and high throughput proteomics

Methods: Tandem Mass Tag TMT labelled, and label free Human Cell lysates were used to evaluate MS utilization, proteome coverage, consistency of results and column carryover using tandem nano-LC fluidics and flowrates with columns ranging from 50 – 75µm inner diameter (I.D.) affording throughputs ranging from 4 to 180 sampler per day (SPD)

Results: The Tandem Direct Injection Workflow permits:

- Maxmimum MS utilization for deep dive and high thoughput – high sensitivity LC-MS workflows
- Viirtual carryover elimination through extensive column washing at no cost to sample throughput

Introduction

Nano and capillary LC-MS (0-1 – 5 µL/min) is widely recognized as the gold standard for deep-dive proteomics workflows, due to its exceptional sensitivity[1,2]. However, this comes at the cost of reduced sample throughput and low MS utilization particularly for direct injection (DI) based workflow configurations.

Here we present a novel tandem direct injection (TDI) workflow which permits offsetting of the gradient runs on the two columns to achieve nearly continuous analyte elution. Coupled with an intelligent instrument method editor (IME) and tailored diagnostic scripts, the workflow also permits comprehensive column washing with no impact on sample throughput. We investigated the capacity of the TDI workflow for both tandem mass tag (TMT) and label-free quantification (LFQ) in both data dependent (DDA) and data independent (DIA) acquisition modes.

Materials and methods

Sample Preparation

Thermo Scientific™ Pierce™ HeLa Digest/PRTC Standard (P/N A47996, 10 µg/vial) was reconstituted by adding 50 µL of 0.1% formic acid (FA) in water with 2% acetonitrile (ACN) or 0.015% *n*-dodecyl-D-maltoside (DDM). The vial was subsequently sonicated for 5 min, followed by aspirating and releasing 10 times with a pipette to fully reconstitute the sample. Thermo Scientific™ TMTpro™ 18-plex labeled HeLa samples were prepared in-house by evenly mixing each channel, followed by dissolution in 2% ACN / H_2O, 0.1% FA prior to analysis.

Hardware and Fluidic Configuration

- Vanquish Neo UHPLC system
- Thermo Scientific™ Vanquish™ Column Compartment N
- Two 2-position 6-port low-dispersion switching valves
- Thermo Scientific™ Vanquish™ Binary Pump N
- Tandem Workflow Kit, Vanquish Neo
- Thermo Scientific™ Nanospray Flex™, ion source
- Tandem Workflow Kit, Vanquish Neo (P/N 6250.1030)
- Tandem Source Kit (P/N B51004433)
- Sonation Double Barrel Oven with Mounting Kit NG (P/N B51003991)
- Thermo Scientific™ Orbitrap Exploris™ 480 or Orbitrap Astral™ MS operated in either DDA or DIA mode

Columns, emitters and connectors, are listed in **Table 1.**

The respective TDI method overviews are given in **Table 2.**

Data Analysis

Acquired .raw files were processed with Thermo Scientific Proteome Discoverer™ 3.1 software using a 2-step SEQUEST™ HT search algorithm and the INFERYS™ rescoring node or CHIMERYS™ DDA node (DDA data) CHIMERYS DIA node (MSAID GmbH), or Spectronaut™ 19 software (DIA data, Biognosys AG). The false discovery rate (FDR) was set below 1% at both the peptide and the protein levels.

Table 1. Columns, Emitters, Connections

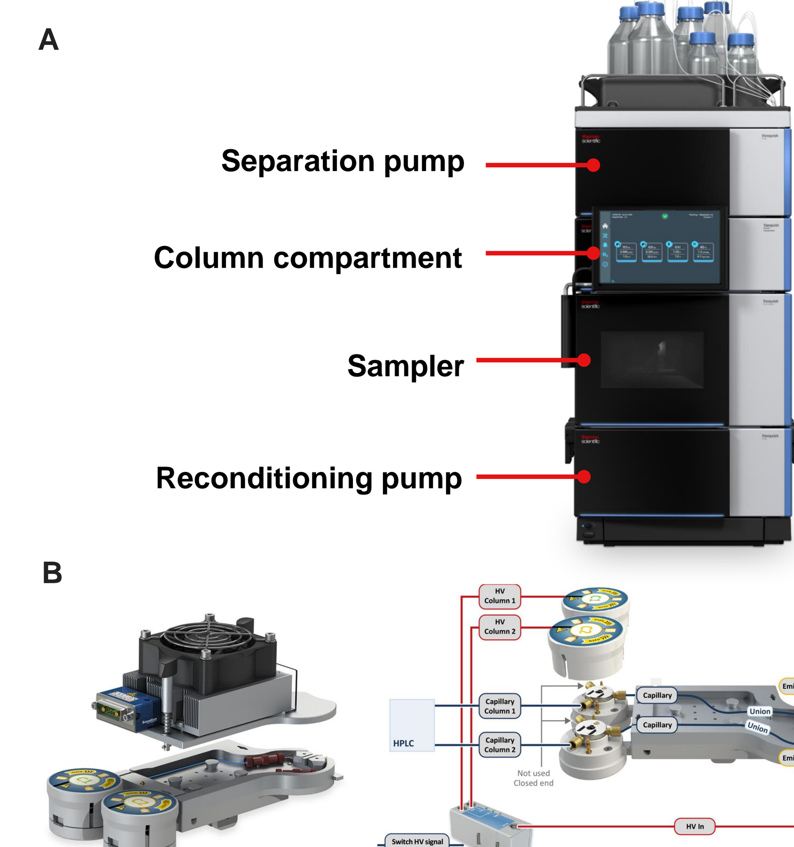
Flow Regime	Separation Column	Emitter	Column- Emitter connector
Nano/ Cap (0.1 – 5 µL/min) using 20µm I.D. Fluidic capillaries	Thermo Scientific™ Single nanoViper™ PepMap™ Neo column, 75µm ID x 75 cm	FOSSILIONTECH 10µm I.D. x 5cm (LOTUS)	MicroTight™ union
	Thermo Scientific™ Acclaim™ PepMap™ column, 75µm I.D. x 15 cm	FOSSILIONTECH 10µm x 5 cm (LOTUS) / CoAnn 30µm I.D. x 5cm	MicroTight™ union
	Thermo Scientific Acclaim PepMap column, 50µm I.D. x 15cm	FOSSILIONTECH 10µm I.D. x 5 cm	MicroTight™ union
	Self Packed 75 µm I.D. x 20 cm	Pulled-tip	-

Tandem Direct Injection workflow - Overview

The Vanquish Neo TDI workflow utilizes 2 LC pumps and 2 columns to eliminate method overhead (**Figure 1A**). The system performs column loading, washing, and equilibration using the reconditioning (lower) pump on column 2 in parallel to chromatographic separation and data acquisition driven by the separation (upper) pump on column one.

The Dual Spray option is employed for flowrates ≤ 1 µL/min. Here the columns are housed in a column which is positioned directly in front of the MS source (**Figure 1B**).

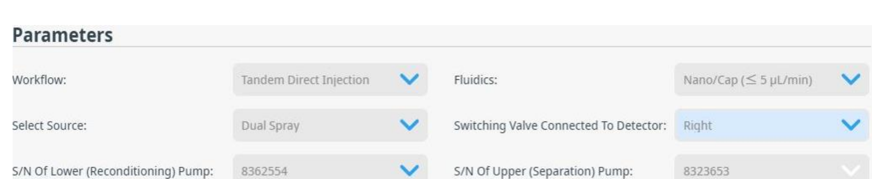
Figure 1 Vanquish Neo TDI solution hardware configuration comprising the LC system stack (A) and Double Barrel Oven (Sonation GmbH) housing the separation columns (B).



Configuring the Dual Spray Tandem nano-capillary Direct Injection workflow

The workflow is configured via the Vanquish System Controller (VSC). Pump roles are assigned according to their physical location within the LC stack. Animated instructions guide the user through the configuring process (**Figure 2**).

Figure 2. Configuring the Dual Spray Tandem nano- and capillary flow workflow on the Vanquish User Interface

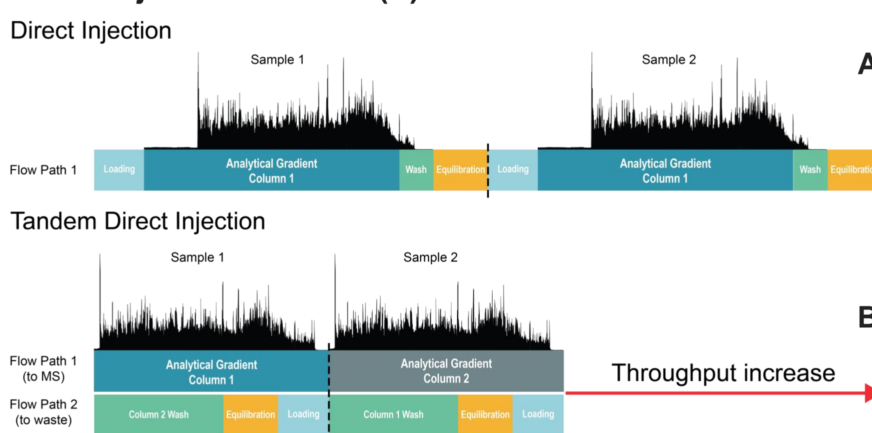


Eliminating Method Overhead with the TDI Workflow

For Direct Injection LC-MS methods, only a portion of the run time can be used for sample data acquisition because each step in the LC run cycle must be performed in sequentially (**Figure 3A**).

For the TDI workflow, the column washing, equilibration and sample aspiration and loading steps are performed in parallel to gradient elution on the other (**Figure 3B**).

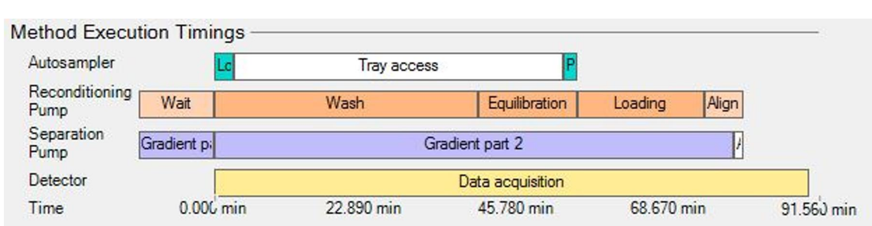
Figure 3. Removing the overhead in the sample run when migrating from the 'single' Direct Injection (A) to Tandem Direct Injection workflow (B)



Intelligent Method Execution

The Instrument method Editor (IME) operates within boundary conditions according to parameters input on the VSC, e.g., column dimensions as well as those garnered from the system diagnostics , e.g., column resistance factors. The IME includes a dynamic graphic of the scheduled duration of the tasks performed by the primary workflow components which varies with the parameter settings input by the user. All complex operations, such as pressure and flow alignment, are handled by the system driver.

Figure 4. Method Execution Scheduling for the 18 SPD TDI workflow



LC Method Overview

Nano-Capillary flow methods were run at flowrates ranging from 0.1 – 1 µL/min using columns of 50 µm and 75 µm I.D. with lengths of 15 cm or 75 cm.

Table 2. Nano-Capillary Method Overview

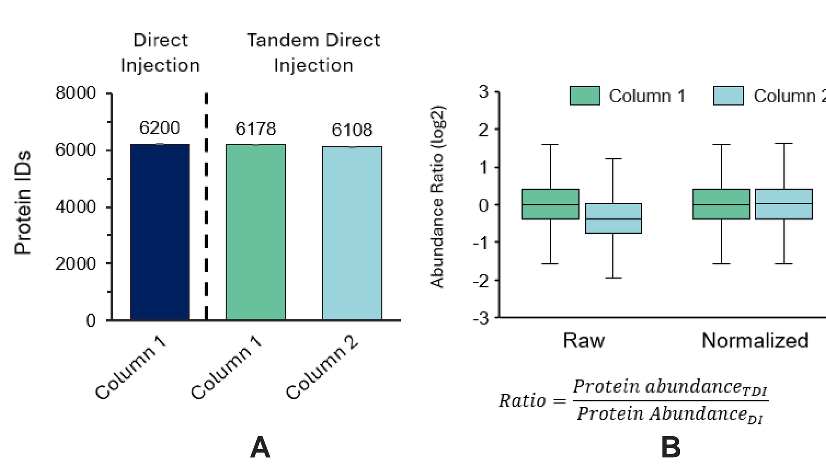
Separation Column	Flow Rate (µL/min)	Throughput (samples/day)	Cycle Time (min)	Elution Window (min)	MS utilization (%)
PepMap Neo 75µm I.D. x 75 cm	0.25	**18**	91.6	86.7	**95**
	0.25	**7.9**	181.6	176.5	**97**
	0.25	**5.3**	271.6	267	**98**
	0.25	**4**	361.6	357	**99**
PepMap 75µm I.D x 15cm	1	**180**	8	7	**88**
	0.8	**120**	12	10.8	**90**
	0.5	**100**	14.4	13	**90**
PepMap 50µm x 15cm	0.1	**72**	20	14.8	**74**
	0.1	**48**	30	24.5	**82**
	0.1	**36**	40	34.5	**86**
	0.1	**28.8**	50	44.5	**89**
	0.1	**24**	60	54.5	**91**

Results

Increased throughput for deep-dive proteomics experiments

Equivalent Proteome coverage was achieved between DI and TDI for 1 µg HeLa digest samples measured on 75 µm x 75 cm PepMap columns using a 90-minute gradient. However, the TDI workflow afforded an increase in MS utilization from 70% to 93% and a 50% increase in throughput to 18 SPD (**Figure 5A**). LFQ based quantification revealed that although column dependent variation was observed, this could be accounted for by normalizing the data using the total peptide amount (**Figure 5B**).

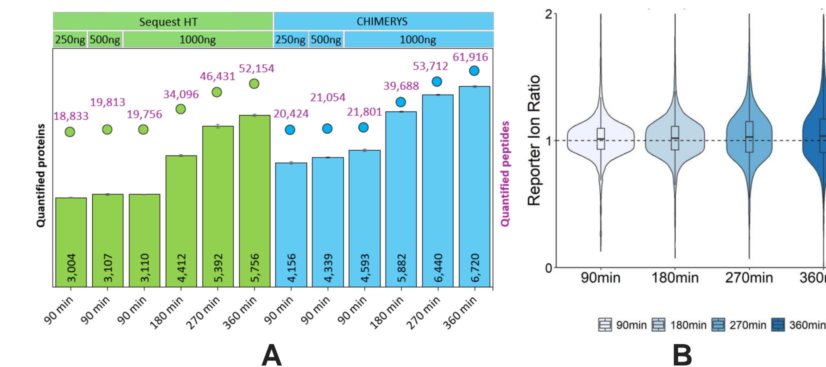
Figure 5. Protein IDs (A) and LFQ abundance level for 90-minute HeLa peptide separations using an Orbitrap Exploris 480 MS



TDI compatibility with TMT labelled samples

The same columns were used to compare TDI vs DI workflows for TMTpro-18plex labelled HeLa digests in single shot DDA experiments on an Orbitrap Exploris 480 MS. >5,700 proteins were quantified from the SEQUEST HT node in a 360-min gradient without pre-fractionation. Adopting the CHIMERYS node increased the number of quantified proteins to >6,700 (**Figure 6A**). Accurate MS2 quantification was obtained for all conditions (**Figure 6B**).

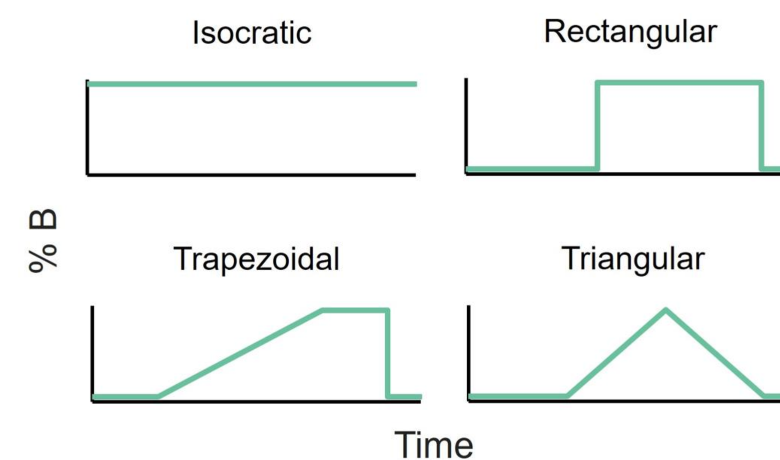
Figure 6. No. of quantified peptides and proteins (A) and quantification accuracy (B) for Tandem based TMTpro 18-plex samples at 250, 500 and 1000ng using 90 – 360 minute gradients



Automated Wash Patterns for Reduced Carryover

The TDI workflow permits extended column washing without impacting MS utilization and sample throughput. The workflow incorporates four pre-programmed wash patterns (**Figure 7**) which can be selected via a dropdown menu.

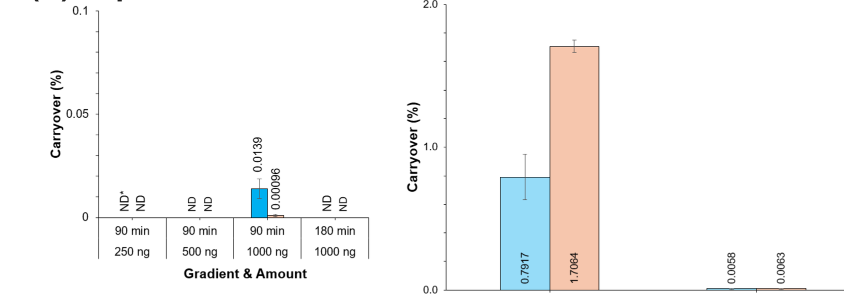
Figure 7. Available pre-programmed wash patterns



Minimizing column carryover with the TDI workflow

Proof-of-principle experiments to determine both the quantitative (quantified peptide area) and qualitative (number of quantified peptides for TMT-DDA (**Figure 8A**) and LFQ-DIA (**Figure 8B**) based applications.

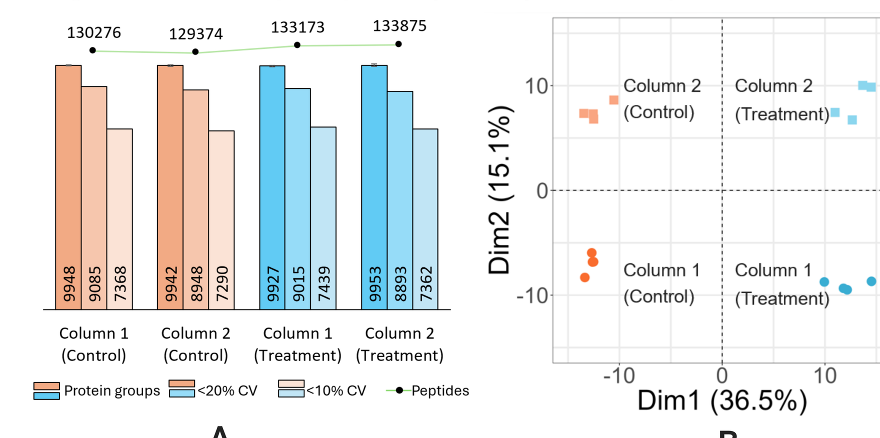
Figure 8. Column Carryover for TMT labelled (A) and LFQ-DIA (B) Experiments



Tandem nano-LCMS for large cohort sample analysis

Analysis of control vs treated (protein degrader) cell lysates with a 96 SPD method yielded approx.10,000 reproducibly quantifiable protein groups on an Orbitrap™ Astral™ MS (**Figure 9A**). Whilst PCA analysis revealed discernable column dependent differences, results show that variation between the control and treatment groups was more significant (**Figure 9B**).

Figure 9. 96 samples per day cell lysate analysis

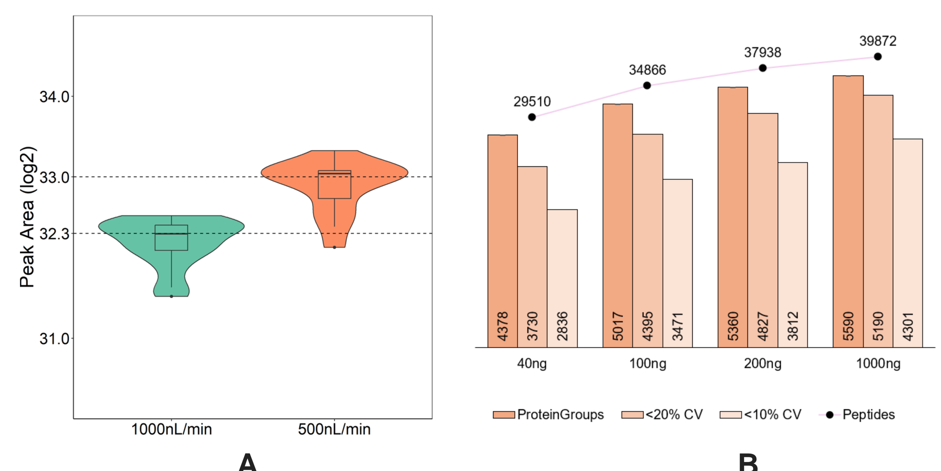


Increased sensitivity and Coverage for High Throughput Proteomics using short (15cm) columns

Trap-and-Elute (T-E) workflows are commonly used for high throughput low-flow analytics as they permit ultra-fast sample loading compared to DI. In TDI the slower sample loading speeds can be accommodated for, enabling deeper coverage at the same throughput. Using a 100 SPD method, the TDI workflows produced 20% more protein groups compared to the 100 SPD Trap-and-Elute based method[4] using a 15 cm x 75 µm ID PepMap column.

The TDI permitted a further 62% increase in sensitivity by halving the flow rate to 500 nL/min whilst maintaining the 100 SPD throughput (**Figure 10A**). This permitted the identification of > 4,300 protein groups from 40 ng digest in DIA mode (**Figure 10B**)

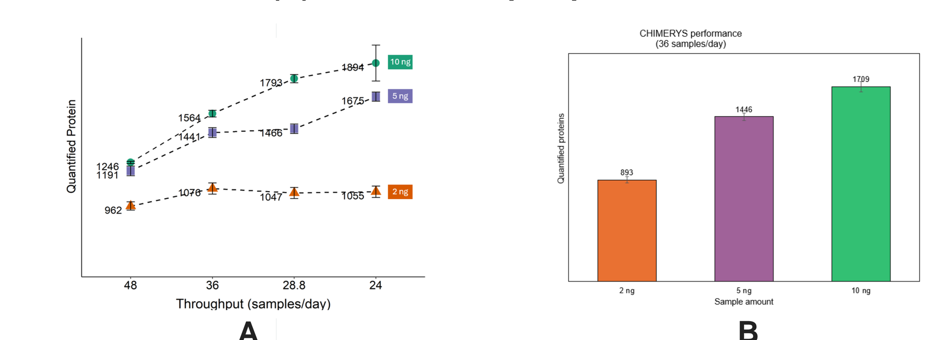
Figure 10. PRTC Peptide intensity (A) and Proteomic Depth and Quantitative Precision with 100 SPD TDI methods throughput in DIA mode on an Orbitrap Exploris 480 MS



Maximizing low-nanoflow LC-MS throughput

Migrating from DI to TDI for the 72 SPD method on 15 cm x 50µm ID columns at 100nL/min yielded a boost from 55% to 74% MS utilization. Using TMT labelled samples, we quantified 1070, 1441 and 1564 proteins from 2, 5, and 10 ng samples respectively (**Figure 11 A**). The quantification performance was enhanced by 9.3% using the CHIMERYS (**Figure 11B**). This equates to 1,260 Hela cells / day for TMT-35plex

Figure 11. Linearity experiment for TMTpro 18-plex using SEQUEST HT (A) and quantified proteins using the CHIMERYS node at 36 SPD (B) on an Orbitrap Explorls 480 MS



Conclusions

A novel tandem "dual spray" direct injection workflow configuration supporting flowrates of 0.1 – 5 µL/min was evaluated for throughput, and MS utilization, data comparability, and carryover.

The workflow offers:

- Intelligent workflow execution and inbuilt instrument diagnostics permitting facile workflow setup, method execution and robustness
- Proteome coverage consistent with the respective single direct injection workflow
- MS utilization ranging from 74 – 99% for elution windows ranging from 7 minutes (high throughput) to 357 minutes (TMT labelled deep dive)
- Extremely low carryover – even for TMT samples - without compromising MS utilization or sample throughput

References

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