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TITLE:

Simple In-House Ultra-High Performance Capillary Column Manufacturing with the FlashPack Approach

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SUMMARY:

Here we present a protocol for the optimized FlashPack capillary column packing procedure. Application of an optimized protocol to a common 100-bar pressure bomb setup allows 10-times faster packing and manufacturing of long ultra-high performance capillary columns.

ABSTRACT:

Capillary ultra-high performance liquid chromatography (UHPLC) is currently a method of choice for the sample separation step in LC-MS-based proteomics. However, capillary columns are much less robust in comparison to their higher flow countertypes. Because of easy contamination and blocking, they often need replacement. That makes them a markedly expensive part of the total LC-MS analysis cost. In-house packing of UHPLC capillary columns saves a lot of money and allows customization. However, the standard packing procedure in the 100-bar pressure bomb works well only for HPLC columns but is too slow for UHPLC sorbents. Here we provide a description of an optimized FlashPack protocol applied to the same 100-bar pressure bomb setup. The method is based on packing from ultra-high sorbent concentration slurry and is developed for in-house manufacturing of UHPLC capillary columns of unlimited length in reasonable time.

INTRODUCTION:

Modern proteomics is based on liquid chromatography-coupled mass spectrometry with the ultra-high performance nano-flow chromatography (50–150 µm column internal diameter (ID))

separation providing the best analysis speed and sensitivity[1]. While numerous commercial UHPLC capillary columns are available, their price makes up a major part of the consumables cost, especially when multiple diverse projects are run in the laboratory and project-specific column contamination is a frequent issue. Besides, packing of columns in-house allows the use of custom experiment-specific sorbents (such as, e.g., polyCAT-A sorbent[2]) and column characteristics not available for buying as a ready-made column.

To cope with that, many laboratories pack capillary columns in-house. However, the common packing procedure with a 100 bar pressure bomb (pressure injection cell)[3] is ill-suited to the UHPLC column packing due to high backpressure of sub 2 μm UHPLC sorbents resulting in a dramatic packing rate reduction in comparison to larger-sized HPLC sorbents. While short UHPLC columns can still be very slowly packed, manufacturing of long UHPLC columns is physically impossible[4].

Standard capillary column packing is done at relatively low pressures—up to 100 bars, and with a very low sorbent slurry concentration. Hence, two possible directions of speeding-up the process are available. It is possible to increase the packing pressure[5]. However, this requires special equipment and, practically, installation of a new method in the laboratory. Another way is to increase the sorbent slurry concentration[6]. High sorbent slurry concentration packing is described in combination with ultra-high packing pressure in a previous publication[7]. However, at 100 bar pressure, which is used in most of the existing packing bombs, higher sorbent concentration results in either packing rate slow-down or outright packing cessation. The effect was recently demonstrated to be due to sorbent clustering at the column entrance, and a simple trick of sorbent cupola destabilization by hammering the column entrance with a magnet bar inside a sorbent vial was suggested[4]. The resulting method, named FlashPack, uses the same 100-bar pressure bomb packing setup. At the same time, minor but critical changes in the packing procedure allow packing from very high sorbent slurry concentration and production of very long UHPLC columns (50 to 70 cm, and longer) in less than an hour, while a short column can be produced in minutes with the separation quality equal to commercial columns of the same parameters[4]. The FlashPack approach was already successfully used in multiple proteomics projects for the preparation of both reverse phase (RP)[8–14] and hydrophilic interaction (HILIC)[2] capillary columns.

Here we describe in detail, the modifications needed for adaptation of the FlashPack approach to the standard 100-bar pressure bomb packing procedure.

PROTOCOL:

The packing protocol consists of five steps (**Figure 1**): 1) Packing station preparation, 2) capillary preparation, 3) sorbent slurry preparation, 4) capillary packing in the pressure bomb, and 5) column packing-up in the HPLC system, cutting up to the size and UHPLC connection installation. The FlashPack optimization requires adjustments to be made in sections 3 and 4 as compared to the common protocol.

1. Packing station assembly

1.1. Prepare a gas tank filled with either nitrogen, helium or argon furnished with a single stage gas regulator with the outlet pressure > 50 bars. Maximum pressure is limited by the pressure bomb compatibility.

1.2. Connect the regulator to the vent valve of the pressure bomb.

1.3. If the pressure bomb is not equipped with an integrated magnetic stirrer, place the bomb on a magnetic stirrer.

1.4. Connect a narrow ID plastic tubing (e.g., 0.13 mm) to the outlet of the pressure bomb and put it into a vessel with water.

2. Capillary preparation

2.1. Prepare a fritted capillary with an integrated glass frit formed from Kasil and formamide[15] or a pulled emitter capillary prepared by a laser puller[16]. The capillary is made 10–15 cm longer than the intended column length.

NOTE: See **Table 1** for the discussion of possible issues associated with different capillary sizes and frit types. **Table 2** contains an example of a P2000 laser puller program for making pulled-emitter capillaries.

2.2. Protect a pulled emitter end with a cut gel-loading pipette tip.

2.2.1. Cut the tip so it fits tightly around the 360 μm OD capillary (it can be moved along the capillary but requires some effort to do that).

2.2.2. Slide the cut pipette tip onto the capillary from the direction of the capillary front end and move it up to the emitter end.

2.2.3. Slide back the protecting tip when the column is spraying. Slide the tip forward to have the emitter end inside the tip when the column is not spraying (even when the column is under the flow, but still not spaying).

3. Sorbent slurry preparation

3.1. Prepare a stock sorbent vial: Place ~50 mg of dry sorbent into a 1.5 mL centrifuge tube. Here, Reprosil Pur C18 is used as an example.

3.2. Add 1 mL of methanol to the sorbent tube.

3.3. To mix it completely, vortex the tube for 10 s using a vortex mixer.

3.4. Sonicate in a sonication bath for 10 s.

3.5. Let the sorbent get soaked thoroughly for 20–30 min. Then, vortex and sonicate it once more.

3.6. Prepare a working sorbent vial. Use a conical bottom vial which fits into the bomb.

NOTE: It can be either another 1.5 mL centrifuge tube or any other vial depending on the particular pressure bomb design. For this experiment, conical bottom screw cap tube cut to the height of the pressure bomb is used.

3.7. Resuspend the sorbent in the stock sorbent vial and transfer 500 µL into the working sorbent vial with a magnet bar of 2 x 3 mm size.

3.8. Add methanol up to ~1 mL to the working vial.

3.9. Let the working vial stand on the table for 10 min for the sorbent to settle by gravity.

3.10. If, after settling, the sorbent layer is below 4 mm, add more of the stock sorbent slurry and wait for the sorbent to settle for another 10 min.

NOTE: The prepared working vial is intended for preparation of multiple columns over months. If the working sorbent vial stays without stirring for more than 2 h, it must be vortexed for 10 s, sonicated for 10 s and settled by gravity. Routinely, the sorbent is resuspended in the morning before packing. Then, it is good for packing for the whole day if there are no long pauses between sequential column packing. If the sorbent in the working vial dries out, add methanol, and run the full sorbent preparation procedure as for the stock sorbent vial (steps 3.2–3.5).

4. Capillary packing in a pressure bomb

CAUTION: Always wear protective glasses when working with the pressure bomb. Do not wear gloves. These severely reduce the sense of touch required for proper handling of small diameter capillaries and lead to mistakes.

4.1. Place the sorbent vial into the pressure bomb and fix all the nuts tightly.

4.2. Start the rotation at 60–100 rpm.

4.3. Insert the fritted or pulled emitter capillary into the bomb: push it to the very bottom of the vial, and then lift it up 2–3 mm and fix the nut.

NOTE: Apply a minimum required force to fix the capillary to avoid capillary and ferrule damage. The best is hand-tightening. If a hex-wrench is used, apply minimum sufficient effort for tightening.

4.4. Check whether the capillary is properly fixed—it must be impossible to move the capillary by pulling it out by hand.

4.5. Very slowly open the pressure bomb valve while keeping the open end of the capillary pointed away from your face.

4.6. Watch the initial steps of the packing process.

NOTE: Immediately upon pressurization, the sorbent fills the capillary and it becomes non-transparent for the whole length. As soon as the sorbent starts to pack inside the distal end, the backpressure increases, the flow slows down and the even sorbent slurry inside the capillary reforms into several sorbent packets separated by sorbent-free gaps. Already packed sorbent is visible as a densely colored continuously growing region.

4.7. Keep the sorbent filled regions to be at least 70% of the capillary length with small sorbent free gaps for the whole duration of the packing process.

4.8. There are several common issues to watch during the packing process, that require on-the-flight setup adjustment to keep efficient sorbent delivery into the capillary.

NOTE: More details on the sorbent-delivery efficiency adjustment are described in **Table 3**.

4.8.1. Issue 1*:* When new sorbent stops entering the capillary while the sorbent already inside keeps moving, follow the steps below.

NOTE: This is the most frequent issue. In most cases the capillary entrance gets blocked by self-aggregating sorbent clusters. Apply the following steps one by one until the sorbent flow is restored, and then skip the rest of the issue-related steps.

4.8.1.1. Increase the rotation speed to 500 rpm and immediately reduce it back to 60–100 rpm Usually, it restores the sorbent flow. Check the rotational speed to be at least 60 rpm for the rest of the packing process.

4.8.1.2. If it does not help, briefly vent the packing bomb and immediately pressurize it back.

4.8.1.3. If it does not help or the blocking happens again, reposition the capillary inside the sorbent layer. The absence of the sorbent can be due to the capillary open end being either too high above the magnet bar, so the column end does not touch it, or the capillary sticking into the vial bottom. First, vent the bomb completely, loosen the nut, push the capillary to the bottom, and then pull it 2 mm back. Fix the nut.

4.8.1.4. If the blocking persists, vent the system, take out the sorbent vial and vortex and sonicate it once more. Check the capillary frontal end for damage under the microscope and cut ~5 mm of the front end if needed.

4.8.2. Issue 2: When the sorbent fills only a small part of the capillary with long empty regions, follow the steps below.

4.8.2.1. Check the rotation speed. If the rotation is too slow, the cupola breaking is not efficient enough—increase the rotation speed to 150 rpm.

4.8.2.2. Also, if the rotation is too fast, slow it down to 60–100 RPM because the sorbent gets resuspended into the larger vial volume and the local sorbent concentration around the column entrance is low.

4.8.2.3. Check the sorbent level. The same issue with little sorbent inside the capillary is observed when there is not enough sorbent in the vial. When the sorbent gets used up, fill the vial with the new sorbent to keep the sorbent layer no less than 4 mm high after gravity-induced settling.

4.9. Keep on packing the column till the target column length plus 5–7 cm is achieved.

4.10. Stop the rotation and very slowly depressurize the bomb.

4.10.1. Open the bomb valve a little and wait for the bubble burst inside the water bottle to subside. Then, open the valve a little wider and again wait for the bubble burst to slow down.

4.10.2. Release the pressure in increments until no gas comes out of the valve.

NOTE: Do not open the valve all the way at once – it will lead to bubbling inside the capillary and the sorbent going back into the vial. If that happens, pressurize the bomb back and wait for the column to get packed again.

4.11. When the gas stops coming out of the vent valve, take the packed capillary out of the pressure bomb.

NOTE: Do not let the column dry out. If not connected to the HPLC system immediately for further packing, put the packed capillary into storage by submerging it whole into 10% EtOH solution. A liquid-tight polypropylene food storage container can be used for capillary storage. Disconnected HPLC columns are stored in the same manner.

4.12. If no further packing is planned, take out the sorbent vial from the bomb and close it tightly. Keep it for further column packing.

5. Packing in the HPLC column

5.1. Connect the packed capillary to the HPLC system *via* an HPLC connection.

5.2. Start the flow at 95% solvent B (80 or 100% acetonitrile, 0.1% formic acid (FA)) targeting 250–300 bar pressure. For 40 cm packed capillary, use the flow rate of 200–300 nL/min.

NOTE: The packing flow rate is 200 nL/min for 40–50 cm column with 100 µm ID packed with 2 µm sorbent. Some other column sizes are listed in **Table 4**. Flow rates for other column lengths and IDs are estimated from the direct proportionality between the backpressure and the column length and cross-section. The exact flow-rate is adjusted to the actual packed length, which is by default longer than the targeted column length. Also note, that the 300 bar pressure targets the physical pressure limit of the HPLC connection. For higher-pressure connections, higher flow-rates up to the connection pressure limit are to be used for faster packing.

5.3. Watch for the loose sorbent inside the capillary getting packed and getting added to the total packed length.

5.4. Without stopping the flow, dip the column body two times into the sonication bath.

NOTE: Do not immerse column ends and capillary connections—only a part of the column body. Sonication step helps to improve column reproducibility, especially for extremely long columns >50 cm long (unpublished data); however, it adds a random chance of breaking the self-assembling sorbent frit inside the emitter end of the pulled emitter capillary and blocking the column completely. While sonication can be universally applied to any glass-fritted columns, we suggest sonicating pulled emitter columns only for the column length > 50 cm.

5.5. When the sorbent bed stops shrinking, dip the column body into the sonication bath two times more without stopping the flow.

5.6. Run the column for an additional 10 min at 300 bars.

5.7. Stop the flow, wait for the pressure to drop to below three bars, and disconnect the column.

5.8. Visually inspect the column for the lack of gaps and discolorations. If any are found, sonication under the flow can be repeated. For critical experiments, consider making a new column.

5.9. Cut the column to the desired length.

NOTE: Properly done cutting is a prerequisite for column efficiency. Make a notch in polyimide coating with the scribe, partially crack the capillary and pull two pieces apart.

5.10. Polish the column front end on a ceramic wafer or with lapping film.

5.11. Reconnect the column to the LC system using a UHPLC connection.

5.12. Start the working flow rate at 2% B depending on the column ID according to **Table 4**. Wait for the pressure to equilibrate and check the column backpressure.

NOTE: The working flow rate is adjusted according to column parameters. For instance, a 30 cm long 100 μm ID column is run at 500 nL/min.

5.13. Ensure that the backpressure is within 5% of the expected value (see **Table 5)**, This confirms that the column is packed properly and is ready to use.

NOTE: The column backpressure is the total pressure in the gradient channel of the HPLC system with the column connected minus the backpressure of the capillaries before the column. At the same time, the values in **Table 5** are arbitrary (they give an arbitrary scale of what to expect). The intra-laboratory similarity of the backpressure from column to column is a more important indicator that everything works properly. The actual absolute backpressure depends on many parameters, such as the sorbent size and characteristics, the capillary ID, manufacturer and batch, the shape of the pulled emitter end or the density and length of the glass frit, the solvent characteristics and the ambient temperature in the room, etc. If the backpressure is too high, see **Table 1** for possible issues.

REPRESENTATIVE RESULTS:

The FlashPack approach is based on the standard packing setup and follows the same packing pipeline. Packing is done into standard fritted or pulled emitter capillaries. The principal optimization lies in the sorbent slurry concentration: the standard method is incompatible with a high-concentrated sorbent suspension used in FlashPack. The result is a fast production method for long UHPLC columns, for example, a column packed for 50 cm length with 1.9 μm sorbent in less than 1 h (**Figure 2**).

To demonstrate the application of the FlashPack approach, a 30 cm 100 μm ID capillary column was prepared (**Table 6**). Packing of ReprosilPur C18 1.9 μm sorbent was done at 60 bars into a 50 cm long 100 μm ID pulled emitter capillary, prepared by a P2000 laser puller. The capillary was packed to ~40 cm in 40 min with some more loose sorbent left inside the capillary. The packed capillary was connected to an HPLC system and run at 300 nL/min with solvent B (80% acetonitrile, 0.1% FA). After two rounds of 5 s sonication, the final packed length was 43 cm. The column was disconnected, cut to 30 cm and connected to the HPLC system using a UHPLC connection. We routinely use 360 μm sleeveless PEEK nut-ferrule and 360 μm stainless steel union. This combination holds at least up to 700 bars if tightened strongly. The manufactured column has a backpressure of 520 bars at 2% solvent B at 500 nL/min, which is consistent with the expected value range (**Table 5**).

As a demonstration of the column efficiency, we used the manufactured 30 cm column to separate 50 fmol of a tryptic digest of cytochrome C protein in a 15 min gradient from 2% to 50%

B. Extracted ion chromatograms showed the peaks to be highly symmetrical with minimum tailing. Average FWHM was around 3 s (**Figure 3**).

FIGURE AND TABLE LEGENDS:

Table 1: Troubleshooting for high working backpressure of the column.

Table 2: P2000/F laser puller program. P2000/F laser puller program for the preparation of pulled emitter capillaries from 360 µm OD 100 µm ID fused-silica polyimide coated capillaries without internal coating at room temperatures 23–25 ˚C.

Table 3: FlashPack-specific packing issues and checkpoints to control during the packing process.

Table 4: Exemplary packing and working flow rates for different column IDs and length.

Table 5: Expected backpressure for a column packed with 2 µm spherical sorbents and run at working flow rate (according to the column ID) in RP solvent system at RT.

Table 6: Exemplary packing of 30 cm UHPLC column.

Figure 1: Capillary column packing scheme. Stages 1 to 3 are preparatory, followed by pressure bomb packing and finished by HPLC packing-up. Stages 3 and 4 are modified for the ultra-efficient FlashPack protocol.

Figure 2: Packing rate for a fritted capillary 100 µm ID with ReprosilPur C18 AQ 1.9 µm at 100 bars in methanol.

Figure 3: Extracted ion chromatograms of tryptic peptides of cytochrome C. Extracted ion chromatograms of tryptic peptides of cytochrome C after separation of 50 fmol in 30 cm long 100 mm ID pulled emitter capillary column packed with ReprosilPur C18 AQ 1.9 µm in a gradient of buffer B (80% acetonitrile, 0.1% FA) and in buffer A (2% acetonitrile, 0.1% FA) from 2% to 40% B in 15 min at 500 nL/min at RT. Detection was performed using a mass-spectrometer. Absolute intensities and extracted m/z ranges for each peptide are shown to the right of the spectra.

DISCUSSION:

In-house capillary column packing is highly popular in large laboratories working on multiple independent projects. However, a common packing method from a low concentration sorbent suspension has major limitations in the speed and is unable to produce long UHPLC columns.

FlashPack is a modification of the standard packing procedure which makes packing from a very high sorbent concentration possible. The theoretical basis of the method lies in the continuous sorbent cupola destabilization at the column entrance for the whole packing duration. The latter is technically achieved by column entrance being continuously hit with a magnet bar. The method

of cupola destabilization is intentionally developed to have the packing setup completely similar to the common packing process, but the trick of FlashPack lies in the details of the sorbent slurry preparation, capillary positioning, and magnet bar usage during the packing process.

The sorbent slurry is prepared as a sediment sorbent layer in a large solvent volume. It is interesting that the pressure bomb-based packing does not require the same packing conditions for column to column. In FlashPack, we never know the exact sorbent slurry concentration around the column entrance. It is impossible to measure and control exactly, as it also changes during the packing process. However, the final columns are still very reproducible[4] irrespective of how the packing was achieved.

The basis for the fast packing lies in the efficient sorbent cupola destabilization. For this reason, it is important to control sorbent entering the capillary and to maintain the optimal cupola destabilization conditions throughout the whole packing duration. There are several possible issues that might prevent efficient sorbent delivery. Some examples of these are sorbent layer resuspension by fast magnetic bar rotation, inefficient cupola destabilization due to either wrong relative capillary to the magnet bar positioning or too slow magnet bar rotation. The issues themselves and how they are to be addressed are discussed in detail in the protocol section.

After the column is packed, the major column parameter to check is the column backpressure. The pressure values listed in **Table 5** provides a reference point to what is expected for one of the popular sub 2 µm bead size sorbent—ReproSil PUR C18 AQ (1.9 µm). At the same time, additional backpressure might be added by the frit or a too narrowly pulled emitter and one should constantly monitor for that. If packing is done into a pulled emitter, we still suggest measuring the expected column backpressure for the particular sorbent in use by packing fritted capillaries first, and then to see whether the self-assembling frit adds too much. For any high-pressure issues, use the guidelines provided in **Table 1** to pinpoint the problem.

In our experience, a packed column without discolorations, gaps, and with the proper backpressure works in 100% of the cases and gives the separation quality close to what can be expected from the column length and sorbent characteristics. A column with discolorations is not guaranteed to work properly but can still give satisfactory results.

Most of the time, if there are any problems with the separation quality, they do not come from the column itself, but rather from other parts of the separation system, namely, pumps, solvents, or connections. Especially potentially harmful is any post-column connections. Bad connection with a dead volume between the emitter and the fritted column leads to major peak broadening and tailing due to very low flow rates in capillary chromatography.

One more important issue specific to the FlashPack approach is that it uses a lot of expensive sorbents in a working sorbent slurry vial. Please remember, that the sorbent slurry in FlashPack is intended for multiple use. Take care of the sorbent. Avoid unnecessary magnet bar stirring to reduce sorbent grinding—remember to stop the rotation as soon as the packing is finished. And

do not leave the open sorbent vial in the pressure bomb to avoid sorbent drying. Though the sorbent can still be used after that, it takes time to remake the sorbent slurry.

The method works equally well for both fritted capillaries and pulled-emitter capillaries. The FlashPack principle increases the packing rate for capillary IDs from 20 to 250 µm (smaller and larger were not tested). It is also applicable to all the sorbents, both fully and superficially porous, we could test (reflecting that the sorbent cupola formation in high sorbent slurry concentration is not limited specifically to RP sorbents). Besides, solvent parameters clearly affect the packing according to their physical and chemical characteristics. For example, less viscous acetone gives even higher packing rate than methanol at the same packing pressure. However, it is also less polar than methanol and reduces sorbent particles sticking to each other. The effect by itself prevents sorbent cupola formation in the beginning of the packing when the flowrate is still high. However, reduction in sorbent particle interaction also leads to less reliable self-assembling frit formation and more frequent pulled-end blocking during the packing. So, while acetone is better for packing of fritted capillaries, it is less suitable for pulled-emitter capillaries, with the methanol as a slurry solvent being slower but suitable for both types of ending. Packing from hexane or dichloromethane (DCM) are extreme cases of switching to acetone from methanol: they are even less polar, so they prevent sorbent cupola formation completely, however they are not fit for pulled-emitter packing at all. Besides, it was noted that extremely low DCM polarity leads to sorbent particles sticking to the internal capillary wall and making a thick layer on it. The layer thickness gradually grows and random local blocks form resulting in the column packed in several parts separated by regions without sorbent. Such effect was observed for C18 Peptide Aeris sorbent.

Another observed issue was YMC Triart C18 sorbent not being suspended in methanol properly, but to form some sort of flakes. However, that does not prevent it from getting packed with the FlashPack and giving very decent separation efficiency (unpublished data). Thus, while not being optimal for some cases, methanol was the most universal solvent to work for all the tested sorbents and columns. It is necessary to mention that we did not yet analyze how different slurry solvents affect column separation efficiency. At the same time, the efficiency of columns packed from methanol is already completely equal to commercial columns for the same sorbents[4].

FlashPack is not the only existing approach to improve the packing rate of UHPLC columns. Fast packing from high sorbent slurry concentration is also possible with the use of ultra-high pressure packing[7]. The advantage of FlashPack is that it is much simpler as it does not require special ultra-high pressure pumps and pressure bombs for sorbent delivery and capillary connections. At the same time, it was demonstrated that the columns packed at extreme pressures can have separation efficiency higher than lower pressure packed columns[17]. And while FlashPack produces columns identical to commercial ones used in the comparison[4], for which we do not know the packing method, it was not yet tested how FlashPack columns stand against ultra-high pressure packed columns.

In summary, the described FlashPack method can be easily adapted to the existing packing protocol in the laboratory with some adjustments made to the protocol, while the setup stays

completely the same. It speeds up the HPLC capillary column packing to minutes' time and allows production of long UHP capillary columns, which is plainly impossible with the standard packing procedure. The overall economy in the time and money for the laboratory by application of the FlashPack approach can be counted in tens of thousands of Euros per year. Additionally, ability to produce UHP capillary columns locally opens the possibilities for experiment customization impossible with the available commercial products.

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DISCLOSURES:

The authors declare that they have no competing financial interests.

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Figure1

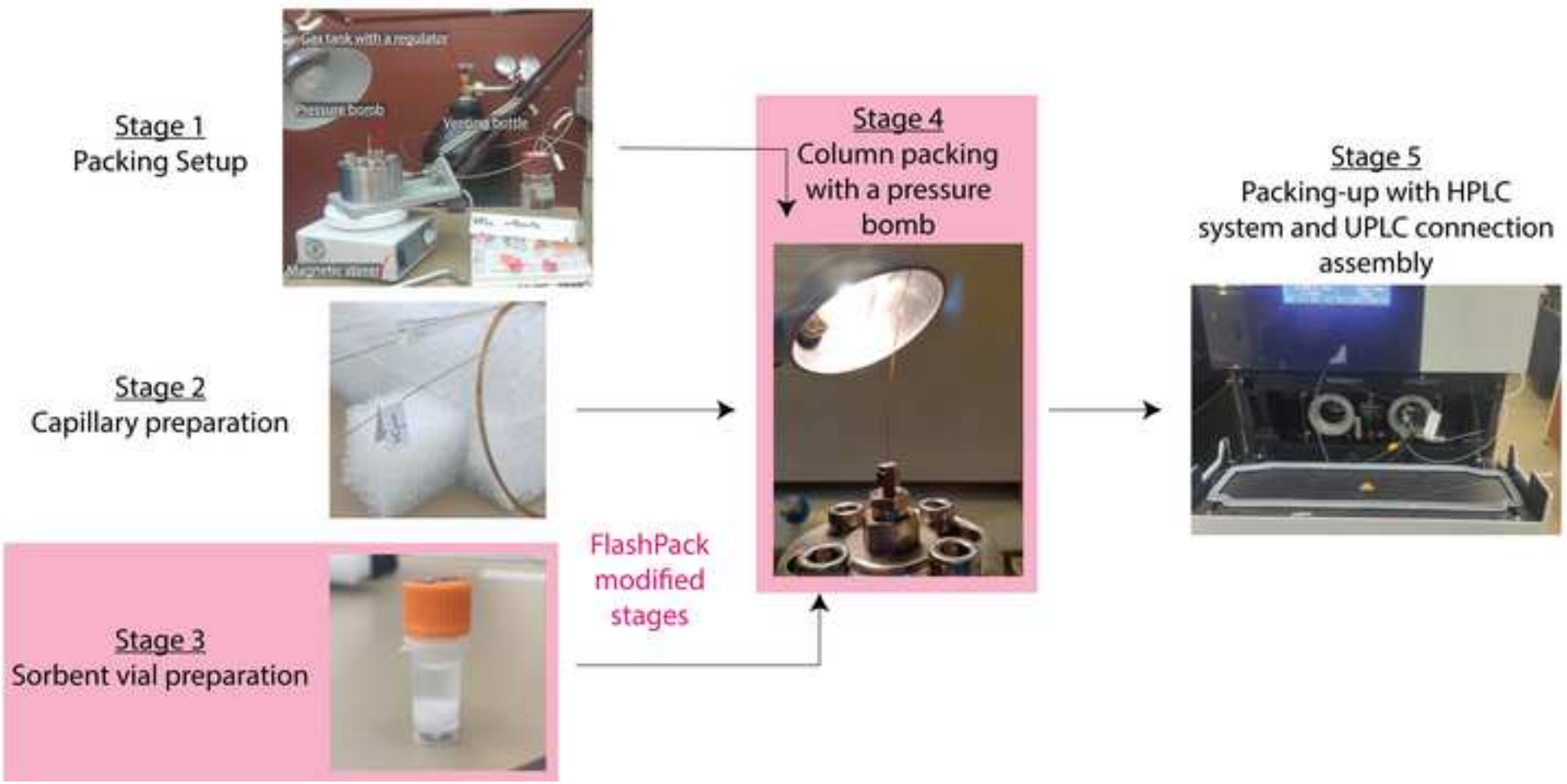

Figure2

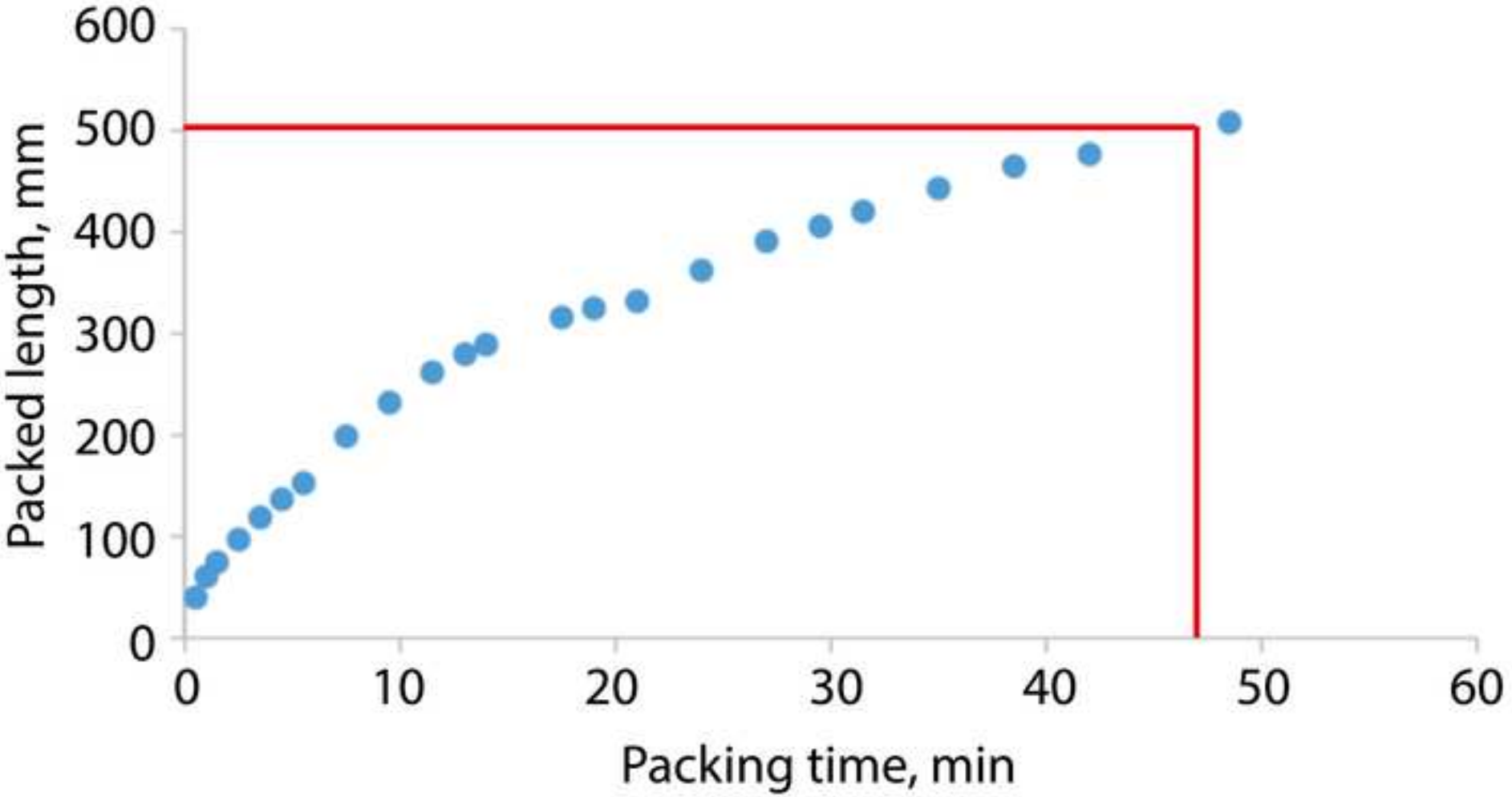

Figure3

Click here to access/download;Figure;Fig3.jpg

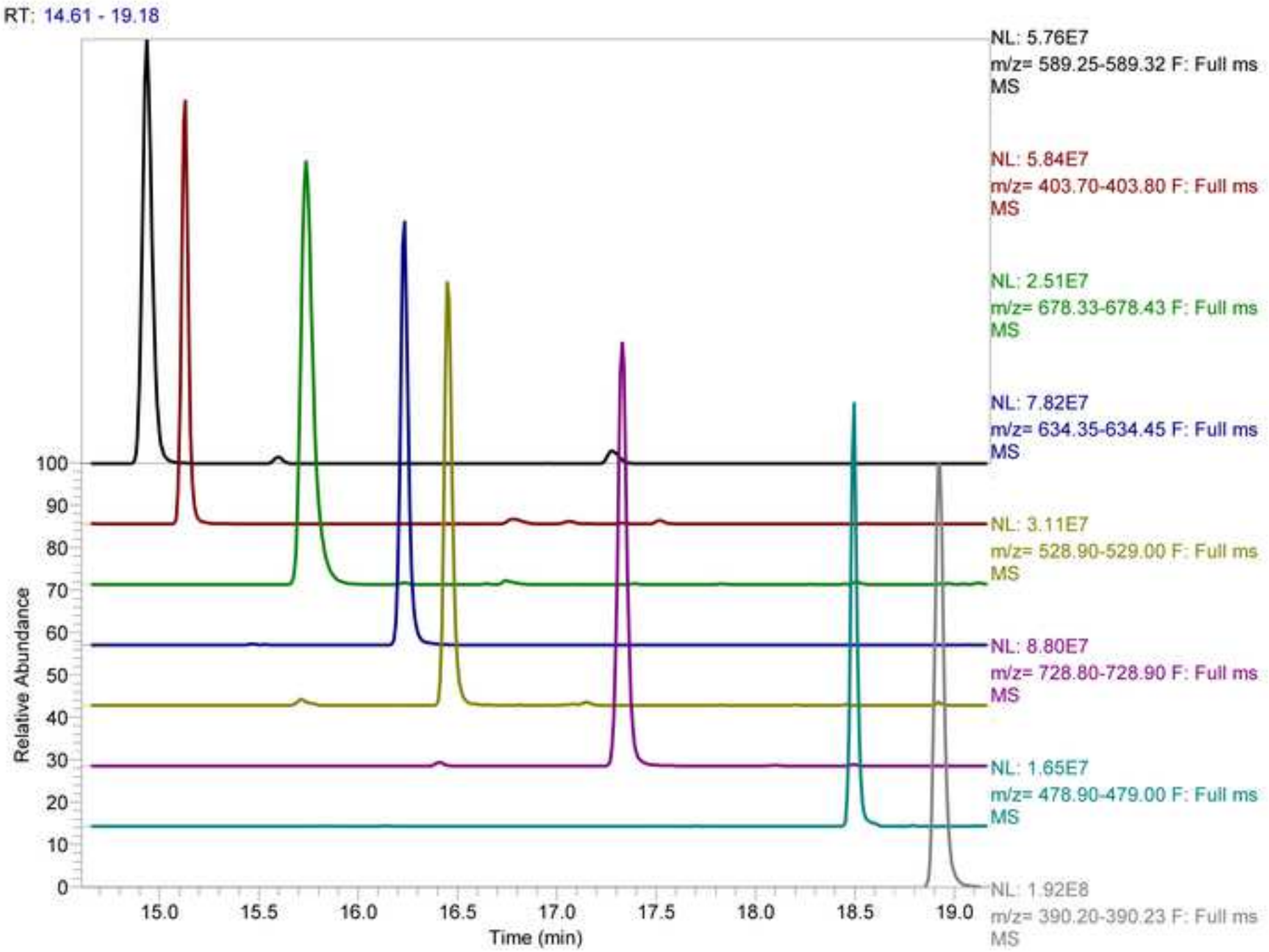

Table1_Troubleshooting for high workir

There are several possible causes for high backp some are specific for pulled emitter columns.

Problem*
A pulled emitter column has a backpressure much higher than expected for the column length.
A pulled emitter column end is blocked by sorbent particles clogging the pulled end (visible under the microscope).

The backpressure is higher than expected from the column length and the effect is reproducible from column to column – happens for both fritted and pulled emitter columns

*Before going to packing troubleshooting, mak
or blocked or restricted connections, capillaries

ıg backpressure of the column

ɔressure, some of which are shared between fritted and pulled emitter columns, while

Possible cause and solution
Possible cause: long pulled region with narrow ID. **Comment:** capillary pulling in a Sutter laser puller implies a part of the capillary with a gradually narrowing ID. If the pulling program is not optimized, a prolonged narrow ID region can be formed which, when packed, has a backpressure much higher than the same length of the original ID capillary. As a result the final pulled-emitter column backpressure is higher than the for the same length of a fritted capillary without narrowing. To reduce the effect, optimize the pulling program to achieve a better hour-glass shape approximation of the pulled end.
Cause: self-assembling frit structure is broken. **Comment:** to make a working column, the sorbent in the pulled end is supposed to form a self-assembling frit –a sorbent wall at some distance from the emitter opening. If the sorbent packs up to the very tip, sorbent particles can partially or completely clog the tip, leading to column blocking. For a very narrow emitter end, a single sorbent particle can block it completely. There are two infallible solutions: - use a specially prepared pulled capillaries with frits integrated into the pulled end (see PicoFrit self-pack columns from NewObjective), which do not rely on the self-assembling frit formation; - adjust the program to get the emitter end to be several times the size of the sorbent particle. In this case, even if the self-assembling frit structure is broken, the column will still work at higher backpressure. The former is good but expensive, and at the moment the item might be out of production.

The latter is practically extremely hard to achieve in a Sutter P2000 laser puller for 75 μm ID capillaries. When pulled normally (the capillary is heated evenly and the walls and capillary ID narrow proportionally when pulled), 75 μm ID capillary separates with the opening around 2-3 μm. Achieving 5-7 μm is possible, but requires a very special laser puller program where the outer capillary layer has a higher temperature than the capillary inside. When pulled, capillary lengthening happens mostly due to outer layer flowing to compensate for lengthening, and the capillary wall narrows faster than the capillary ID. In the end, the emitter separation happens with larger opening diameter. However, such programming is individual for each P2000 instrument. Today, Sutter makes such programs at the factory and provides them for each new P2000 laser puller instrument installed. However, old instruments do not have such programs (though it was possible to send the instrument to the Sutter factory to make such programs), and from our experience, we could not make a required program by ourselves. So, we suggest using 100 μm ID capillaries instead. They have thinner walls from the beginning which separate with the opening around 3-5 μ m under normal pulling conditions (an example of the pulling program is in **Table 6**). Thus, they are much less prone to clogging due to separate particles getting into the column end – separate particles just flush out of the capillary while the major particle front forms the self-assembling frit. At the same time, to achieve exactly the same sensitivity for 100 μm ID columns in comparison with 75 μm ID columns, it might be necessary to increase the peptide loading amount ~ 1.5-2 times.

Independently of the capillary used for the column preparation, avoid the factors leading to the self-assembling frit breaking:

- do not immerse pulled emitter end into the sonication bath during packing;
- avoid column end drying: do not leave the column without flow when connected the LC system; fill the emitter end protecting tip with water before disconnecting the column from the LC system and place the column into the storage box filled with ethanol-water solution as soon as possible;
- avoid dramatic pressure drops, e.g. valve opening, tubing connection disassembling under pressure.

Cause: the sorbent in the working vial is ground by the magnet bar with the accumulation of small sorbent debris.

Comment: The cause can be pinpointed based on the following indications:

- gradual rise of the columns pressure in time with each new packed column batch with the use of the same sorbent vial for packing: e.g. columns produced two weeks ago were fine, last week columns had the pressure a little higher, today it is even higher;
- very long usage of the same working sorbent vial for packing;
- the working sorbent vial was forgotten in the pressure bomb with the rotation turned on for a prolonged period of time.

To avoid that problem, prepare fresh working sorbent as a first-step in packing troubleshooting. The amount of the sorbent in the working vial is cheaper than the time and resources spent on finding the problem.

e sure, that the overpressure is not due to an incorrectly working pump, wrong buffers and emitters, but is really a column problem.

Table 2. P2000/F laser puller (Sutter, USA) program* for the preparation of pulled emitter capillaries from 360 µm OD 100 µm ID fused-silica polyimide coated capillaries without internal coating at the room temperature 23-25°C.

First pulls (the instrument is cool):

Line	Heat	Velocity	FIL/PUL/DEL**
1	300	25	-/-/128
2	300	25	
3	290	25	
4	250	30	

5-10 pulls later (the instrument is warmed up):

Line	Heat	Velocity
1	270	25
2	260	25
3	250	30
4	250	30

*The actual program must always be adjusted and tested locally before column preparation, since the exact pulling depends on the capillary characteristics (which change from batch to batch), laser focus exact positioning, cleanness of the mirror, laser power, room temperature and humidity, etc.

**FIL (filament) and PUL (pulling) parameters are empty and DEL (delay) is 128 for all lines.

The general idea behind obtaining a bell-like shape is gradually decreasing the temperature along with the capillary wall thinning while at the same time increasing the velocity.

Table3_FlashPack-specific packing issues to control during the packing process

Packing stage	What to check
Checkpoint 1	Upon pressurization, the capillary is filled with clear solvent instead of the sorbent slurry.
Checkpoint 2	The pulled emitter capillary is filled with the sorbent slurry, but no liquid comes out of the pulled end and the packed region does not grow.
Checkpoint 3	The sorbent already in the capillary is moving but no new sorbent is entering the capillary *(Packing Issue 1).*

	The sorbent-containing regions fill only small part of the capillary with long “empty” regions *(Packing Issue 2).*

Solution
The capillary front end is not inside the sorbent layer. Vent the bomb and reposition the capillary 2-3 mm above the vial bottom inside the sorbent slurry layer.
The pulled end is blocked. Take a new capillary for packing.
The capillary entrance is blocked by sorbent cupola. 1. A sorbent cupola formed around the column entrance. Increase the rotation speed to 500 RPM and immediately reduce it back to low speed. Increase the rotation speed for the rest of the packing process to 150 RPM. 2. If it does not help or the blocking occurs again some time later: The capillary front end possibly sticks into the vial bottom. That creates a restriction and the sorbent cupola is easily formed. Vent the bomb and reposition the capillary 2-3 mm above the vial bottom. 3. If the capillary entrance blocking persists - it happens again and again: - sorbent beads are in clumps which easily aggregate into the cupola around the column entrance. Take out the sorbent vial and run the “vortex-sonicate-settle” cycle to separate the beads;

- the column entrance is damaged so that it is narrowed or partially clogged with debris or dirt. Sorbent cupola forms the easier, the smaller the front end opening size. Check the capillary entrance for damage - if any doubt, cut the capillary front end 5-10 mm.
Inefficient cupola destabilization and/or too low sorbent concentration around the column entrance. 1. Check the rotation speed – adjust it to 60-150 RPM. 2. Reposition the capillary 2-3 mm above the vial bottom. 3. Check the sorbent level in the vial – it must be >4 mm high when gravity-settled. If necessary, add more sorbent.

Table4

Click here to access/download;Table;Table4 Exemplary packing and working flow rates.xlsx

Column ID, µm	HPLC packing-up flow rate*, nL/min		
	15 cm	30 cm	50 cm
50	250	125	65
75	560	280	140
100	1000	500	250
150	2250	1125	560
200	4000	2000	1000

* Packing flow rate can be increased or decrease
250-300 bar. The values in the table are for 2 µm
particles or higher temperature allow higher flow
and lower temperature).

** Listed working flow rates are efficient for most
require other eluent velocity to achieve maximum

Working flow rate, nL/min (flow rate targets efficient eluent velocity)**
125
280
500
1125
2000

d depending on the actual backpressure targeting sorbents and column running at RT. Larger sorbent rates for packing (the reverse for smaller sorbent

of the sorbent sizes, however some sorbents may separation efficiency.

Table 5. Expected backpressure for column packed with 2 µm spherical rate (according to the column ID) in RP solvent system at RT.

Column length, cm	Expected backpressure, bar*	
	2% B (80% acetonitrile in water)	95% B
15	220-250	140-180
30	450-500	250-280
50	750-830**	400-480

*~10% higher backpressure is expected for pulled-emitter columns with pr comparison to glass-fritted columns (e.g. 450 and 500 bar backpressure fo pulled emitter columns correspondingly). In case of non-optimal pulled-em end of the pulled emitter, the backpressure can increase up to almost com Table 5 for discussion on the pulled end production)

**For columns longer than 30 cm heating is recommended to reduce the b 600 bar. Higher backpressure leads to much faster valve rotor and stator w

sorbents and run at working flow

operly fashioned pulled emitters in
r 30 cm long glass-fritted and
nitter shape and a long narrowing
plete column blocking (see also

backpressure during the run to 500-
vear.

Table 6. Exemplary packing of 30 cm UHPLC column

	Target column length	30 cm
Step number	**Parameter**	**Value**
1. Pressure bomb setup	Packing pressure:	60 bars (nitrogen)
2. Capillary preparation	Capillary:	50 cm 100 µm ID 360 µm OD pulled emitter capillary
3. Sorbent slurry	Sorbent:	ReprosilPur C18 AQ 1.9 µm (Dr. Maisch)
4. Packing in a pressure bomb	Packing time:	40 min
	Packed length:	40 cm
5. Packing in HPLC system	Packing flow rate:	300 nL/min (solvent B – 80% acetonitrile, 0.1% FA)
	Packed length:	43 cm
	Final column length:	30 cm
Column working parameters	Working flow rate:	500 nL/min (99% solvent B)
	Working pressure:	275 bar

Editorial comments:

Text
1. The editor has formatted the manuscript to match the journal style. Please retain and use the attached version for revision.
2. Please address all the specific comments marked in the manuscript.

The issues in the text are addressed as “Answer:” part in each comment.

3. Please remove embedded tables and embedded figures from the manuscript text. All tables must be uploaded as .xlsx files individually and all figures must be uploaded individually with all panels combined into one image file.

Done and uploaded separately.

Video:
Throughout the video, whenever the images are on the screen it has a white background but there is too much black space which can be fixed by adding a white background with cross dissolve/film dissolve/fade effect at the start and end of the background.
Subtitles are not necessary and can be removed.
9:19 Please do not show the commercial term Thermo scientific.
Format:
• Please consider capitalizing first letter of all important words in Title/Chapter Card, also increase the duration of all Title/Chapter Card to atleast 4 seconds.
Video Comments:
• 1:35 - 2:03 Please align the visual to the center of the video frame vertically and horizontally.
• 1:44 For a couple of frames the footage feels dull, Please remove the effect if used here. Please consider removing the transition-cross/film dissolve effects as, for a few frame it's getting fade out.
• 6:48 Please use a cross dissolve between the footages as a transition or remove the gap between the footages so that there is not black frame visible.
• 5:11 Please remove the time remapping here It might give funny impression (As the human hands can’t move this fast). Footages seems to be fast forwarded, please consider keeping it at a level where it's not noticeable and trim the footage to avoid creating more space wihtout narration as leaving in footage that has no accompanying instruction can slow down the pacing and cause 'drag' with the audience.
• 6:48 - 6:55 As per JoVE criteria, "Visual content should fill the entire video frame, therefore black bars on the sides of the video (“letterboxing” and “pillarboxing”) are not permitted". Pleaes consider fixing it by scaling/positioning of the footages. Note: Please make sure to check whole video and address it by scaling/positioning of the footages as, video may have more parts which can be addressed.
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• Capitalize all the Title cards
• 0:46, Consider removing the middle name and PhD from the author, Titles (Dr., Prof., etc.), degrees, or/and credentials (PhD, MD, etc.) should not appear alongside names in Lower Thirds, Consider using a short form of the Institute, “IBCh RAS” Common and well-known abbreviations for institutions should be used when available . Please consider removing "PhD",

"Shemyakin-Ovchinnikov" & "Moscow, Russia" words from text card/lower third.
• Visibility for in-video text can be improved. Consider adding dark & less transparent background to the text (excluding subtitles) for better visibility.
• 3:45 - 3:52 Please change the "," to "." in "1,5 mL" subtitle text.
• 3:58, 4:35, 7:52 For one frame/less than a second subtitle text is getting totally black (including background), please consider addressing it.
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• 13:00-13:29 Consider using a white background scaled to fit to full screen. Please consider adding a full framed white background so that the black/blank spaces are invisible to the viewers.
• 13:28 White bars on the left and right seems to appear without any transition whereas rest is appearing with dissolve/fade. Please consider adding cross dissolve to the white bars/background so that everything on the screen appears with same effect and on the same time.
Audio Comments:
• Audio levels are exceeding -6dB level in some places, please consider maintaining audio levels between -6dB to -12dB by setting the maximum audio gain to -6dB.

All the issues were addressed. There are several questions remaining:

>>Subtitles are not necessary and can be removed.
All subtitles should be removed? It is probably ok to remove subtitles in the introduction section, while keeping subtitles for the method section. For the moment all the subtitles are still there.

Video Comments:
>>• 1:35 - 2:03 Please align the visual to the center of the video frame vertically and horizontally.
If the subtitles here are retained, the subtitle text will overlap the images. Otherwise, subtitles can be removed from the introduction section, then the visual center can be centered for this particular frame. For the time being added white background everywhere and kept the subtitles.

Graphics & Text:
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Changes are made. Now it is a smaller picture inside a larger picture. Is it ok now?