# Trifluoroacetic acid performance of the Vanquish Flex Binary UHPLC system

**Authors**
Christoph Strobl, Matthias Krajewski, and Remco Swart
Thermo Fisher Scientific, Germering, Germany

**Keywords**
Trifluoroacetic acid, baseline ripples, mixer volume, stroke volume, flow consistency

## Goal

Mixing ripple free TFA applications using the Vanquish Flex Binary UHPLC system

## Introduction

Trifluoroacetic acid (TFA) is the most common ion-pairing agent used in reversed-phase (RP-) UHPLC for peptide and protein separations. It lowers the pH and modifies the interaction of the molecules with the stationary phase to control selectivity and thus enhance separations. Common conditions for peptide and protein separations include linear and shallow, low organic to high organic, LC gradients where the mobile phase is composed of water and acetonitrile containing approximately 0.1% TFA. Typically, the analytes are detected with a UV detector at 210–220 nm for peptide bonds, as well as at 280 nm for aromatic amino acid residues.

However, under these analytical LC conditions TFA shows some undesirable effects. TFA strongly absorbs UV light below 250 nm, depending on the water/acetonitrile ratio,[1] resulting in a strong shift in baseline during gradient elution. In addition, TFA is retained on RP columns causing the TFA concentration of the mobile phase within the column to fluctuate with varying organic solvent concentration. In the case of incomplete mixing or fluctuating mobile phase content, the dynamics of TFA equilibrium in the column are disturbed causing a strong amplification of mixing noise. Because TFA absorbs 50–100 times stronger than water or acetonitrile in the UV range, significant baseline ripples are observed.[1]

As a consequence, the TFA associated baseline ripples can significantly increase the limit of detection (LOD) for analytes. The LOD is defined as the lowest analyte concentration that can be detected over baseline noise and is usually expressed as the concentration at a signal-to-noise ratio of at least 3:1. Those baseline ripples can mask the detection of low concentrated and harmful impurities.

A solution to reduce baseline ripples is to use larger mixer volumes,[2] which also increase the gradient delay volume (GDV) of a LC system. However, by increasing the mixer volume, the separation is delayed, which translates into longer LC run times and therefore limits sample throughput per day. When throughput is a concern, UHPLC systems with small GDVs, and therefore with small mixer configurations, are the preferred option.[3]

When faced with a challenging TFA application that requires high throughput ("small mixer volume required") and low LOD ("large mixer volume required"), one fundamental requirement is that the pump flow must be extremely consistent to avoid fluctuations of TFA concentration.[4]

In this context, the new Thermo Scientific™ Vanquish™ Flex Binary system was compared to the Thermo Scientific™ UltiMate™ 3000 Binary RS system in TFA mixing ripples using the standard configuration of the mixer volume of 200 µL (Figure 1). The standard setup of the Vanquish Flex Binary UHPLC system already showed a significant improvement in TFA baseline ripples due to new technologies used in the Vanquish UHPLC platform. However, more adjustments can be made to reduce the TFA mixing ripples further.

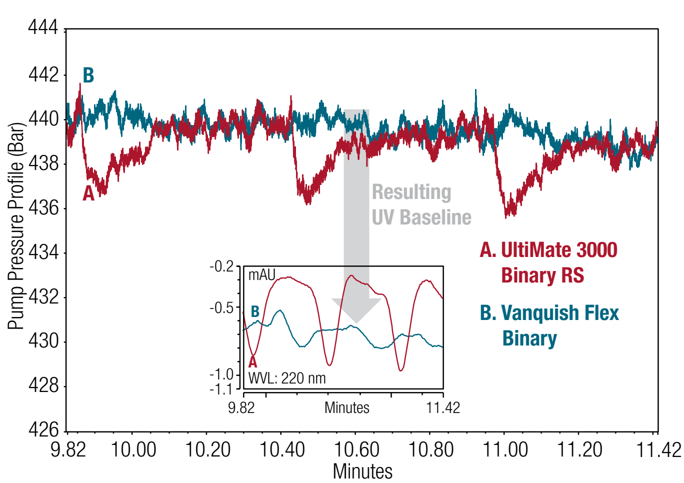


**Figure 1. Pump pressure profiles and UV baseline absorbance of the Vanquish Flex Binary system and the UltiMate 3000 Binary RS system.** The standard mixer volume of 200 µL was used for both systems.

To improve chromatographic separations for TFA related applications, this technical note will focus on three specific aspects of the pump that contribute to baseline ripples and what can be done to minimize their effects:

1. Mixer volume

2. Stroke volume

3. Flow consistency

## Experimental

### System equipment

- Vanquish Flex Binary system consisting of the following:
  - System Base (P/N VF-S01-A)
  - Binary Pump F (P/N VF-P10-A)
  - Split Sampler FT (P/N VF-A10-A)
  - Column Compartment H (P/N VH-C10-A)
  - Diode Array Detector HL (P/N VH-D10-A)
  - Flow Cell, 10 mm Thermo Scientific™ LightPipe™ (P/N 6083.0100)
- UltiMate 3000 Binary RS system consisting of the following:
  - Solvent Rack with Degasser (SRD-3600; P/N 5035.9230)
  - Binary High-Pressure Gradient Pump (HPG-3400RS; P/N 5040.0046)
  - Thermostatted Autosampler (WPS-3000TRS; P/N 5730.0000)
  - Diode Array Detector (DAD-3000RS; P/N 5082.0020)
  - Flow Cell, semi-micro, 7 mm, 2.5 µL, SST (P/N 6082.0300)
- Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software, version 7.2 SR4

### Consumables

*Reagents and chemicals*

- Ultra-pure lab water, 18.2 MΩ·cm at 25 °C
- Acetonitrile Optima™ LC/MS grade (Fisher Scientific P/N A955-212)
- Trifluoroacetic acid, LC/MS grade (Thermo Scientific P/N 85183)

| LC conditions | |
|---|---|
| Column: | Thermo Scientific™ Accucore™ C18, 2.6 µm, 2.1 × 100 mm (P/N 17326-102130) |
| Eluents: | A. Water containing 0.1% TFA |
| | B. Acetonitrile containing 0.085% TFA |
| Gradient: | 0–0.1 min: 5% B,<br>0.1–30 min: 5–55% B,<br>30–30.02 min 55–100% B,<br>30.02–32 min 100% B,<br>32–32.02 min 100–5% B,<br>32.02–37.1 min 5% B |
| Flow Rate: | 0.6 mL/min |
| Pressure: | From 300 to 450 bar |
| Temperature: | 25 °C |
| Injection: | 1.0 µL |

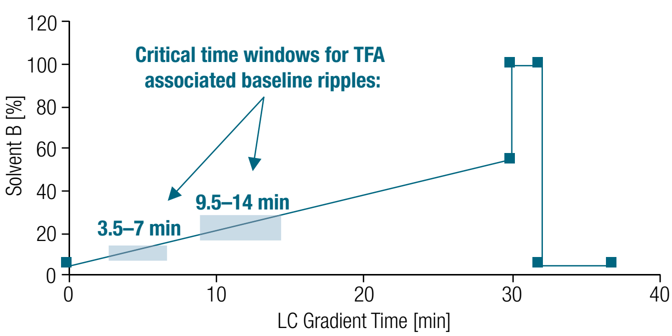


**Figure 2. LC gradient with critical time windows for TFA associated baseline ripples highlighted.**

## Results and discussion

### Effect of mixer volume

The pump mixer receives the volume period of solvent A and B delivered by the pump. As a theoretical experiment for better illustration, let us think of the two pump blocks as conveyor belts that run at different velocities, *v*, representing different flow deliveries (Figure 3A), for example:

Conveyor belt A: 4 *v*, e.g. 80% water

Conveyor belt B: 1 *v*, e.g. 20% acetonitrile

This reflects starting conditions for the LC run of 20% solvent B. The parcels (red: from pump A; yellow: from pump B) represent the “disturbances” within the consistent pump flow. The distance between the parcels (here: 1 *m*) is the volume period, which is 80 µL for the Vanquish Flex Binary system (Figure 3A). The parcels on conveyor belts A and B are transferred to a larger conveyor belt, corresponding to the mixing of solvent A and B at the mixing point of a high pressure gradient pump. The speed of the large conveyor belt is the sum of both velocities (here: 5*v*), which corresponds to the volume period that is the sum of all compressed solvent volumes A and B at the mixing point (here: 100%). Subsequently, each parcel (representing the disturbances from the pump) that is delivered from its conveyor belt will accelerate:

Red parcels from pump A:

$$v_A = v \cdot \frac{100\%}{80\%} = 1.25v$$

Yellow parcels from pump B:

$$v_B = v \cdot \frac{100\%}{20\%} = 5v$$

The increase of each velocity results in larger distances between parcels on the larger conveyor belt, meaning larger volume periods (Figure 3A):

Red parcels from pump A:

$$distance_A = \frac{5v}{4v} \cdot 1m = 1.25m$$

Yellow parcels from pump B:

$$distance_B = \frac{5v}{1v} \cdot 1m = 5m$$

The assumption that the distance of 1 *m* on the conveyor belt, corresponding to the volume period of 80 µL in the pump, results in the following single volume period for each solvent after the mixing point:

(1) $v_A = 1.25 \cdot 80\ \mu L = 100\ \mu L$
(2) $v_B = 5.00 \cdot 80\ \mu L = 400\ \mu L$

$V_A$: Volume period A (after mixing point)

$V_B$: Volume period B (after mixing point)

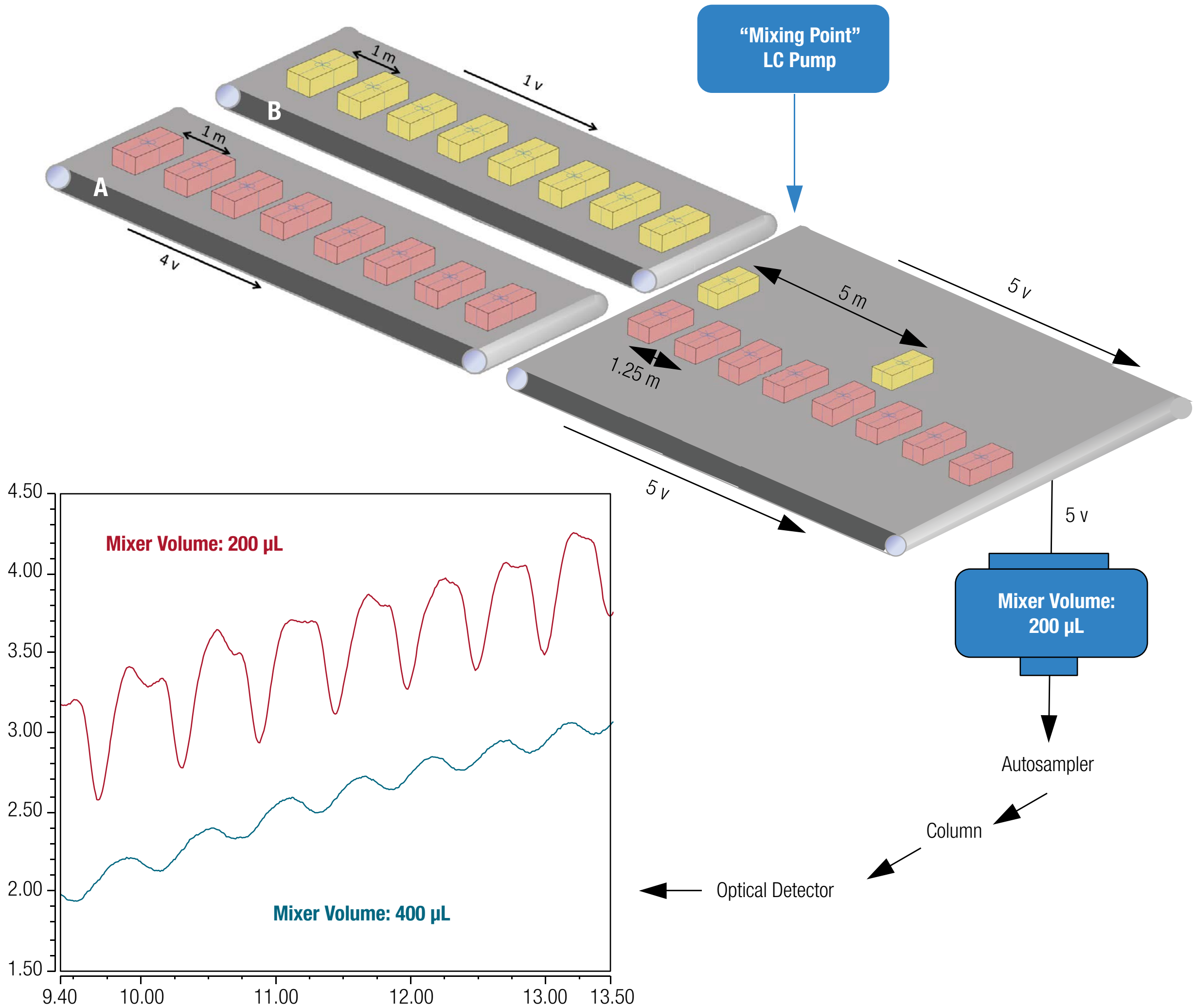


**Figure 3. The volume period of the LC pump.** (Top) Illustration of solvent flows A and B (represented as conveyor belts A and B) converging at the mixing point and a third conveyor belt to the mixer. The volume period of the LC pump is depicted as the space between parcels (e.g., 1 m) on the conveyor belts. (Bottom) Influence of the mixer size on baseline ripples at constant volume period using the UltiMate 3000 Binary RS system.

To achieve good mixing, the volume period of the disturbance needs to be smaller than the mixer volume. In the example, this is the case for A (100 µL < 200 µL) but not for B (400 µL > 200 µL). As a consequence, incomplete mixing of solvent B within the total flow occurs resulting in fluctuations of mixing concentrations. When using solvents with TFA and UV detection, the fluctuations generated by the pump are detectable and are amplified by the retention of TFA on the column (Figure 3B). Shallow LC gradients, typical for TFA applications, have very large volume periods for solvent B since the initial level of solvent B is very low and increases very slowly during gradient formation (typically < 2% B per min). This slow increase results in long time segments in the LC run where baseline ripples can appear (Figure 2). Therefore, even large pump mixer volumes cannot completely mix this large volume period. As a result, TFA associated baseline ripples can be significantly reduced, but not resolved, by using a larger mixer volume of e.g. 400 µL (Figure 3B) at the cost of increased system GDV.

## Effect of stroke volume

The volume of each compressed solvent volume A and B depends on the stroke volume of the pump pistons. In the conveyor belt illustration, the distance between two parcels (the volume period) was assumed to be 1 m, or 80 µL (Figure 3A). To experimentally achieve a small stroke volume, the fixed mixing point of the pump (Figure 3A) can be replaced by a network of different arranged Thermo Scientific™ Viper™ capillaries via mixing T's (Figure 4). The volume period A flows through capillaries with an inner diameter wider than that of the volume period B. At the same time, the split volumes of the volume period B flow into the volume period A, at four different positions. Subsequently this assembly of capillaries causes the splitting of flow B (acetonitrile) to simulate a reduced volume period B of 16 µL, whereas the volume period A remains at 80 µL.

This would correspond to a reduction of the parcel distance from 1 m to 0.2 m in this theoretical experiment. As a result, the volume period B behind the mixing point decreases significantly from 400 µL (see equation on page 3) to 80 µL. Now complete mixing of each solvent within the total flow can be achieved at comparable GDVs using the UltiMate 3000 Binary RS system with the 200 µL mixer (Figure 5A). Residual baseline ripples are reduced significantly, but not resolved, with this flow splitting approach (Figure 5B). In TFA based applications, the extent of volume period A and B divergence is so pronounced that the successful use of small stroke volumes has its limitation. In the example, using a 16 µL stroke volume and 5% or less solvent B as the LC starting condition will translate into a volume period B of 480 µL or higher, which cannot be completely mixed using a 200 µL mixer.

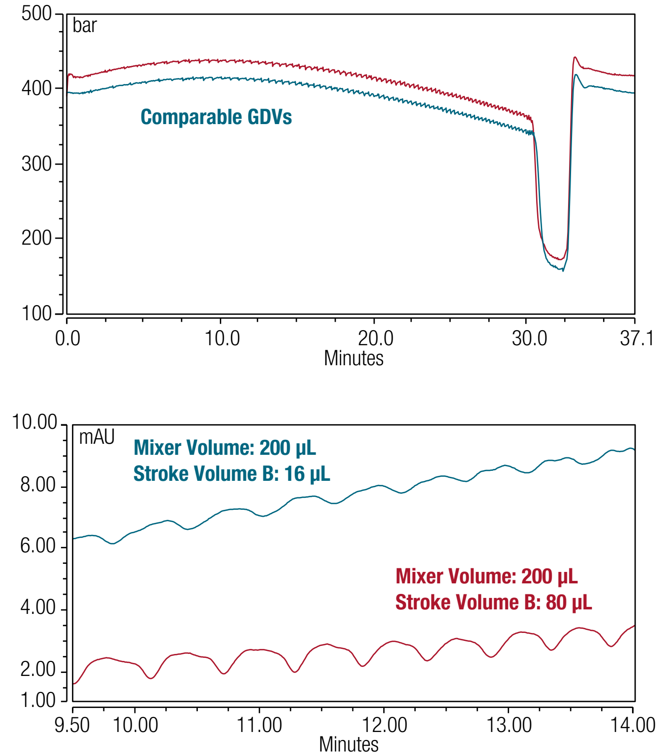


**Figure 5. Effect of small stroke volume.** (Top) Comparable GDVs between the UltiMate 3000 Binary RS system with 200 µL mixer (red color) and with mimicked mixing point. (Bottom) Influence of small stroke volumes on TFA baseline ripples at fixed mixer volume (blue color).

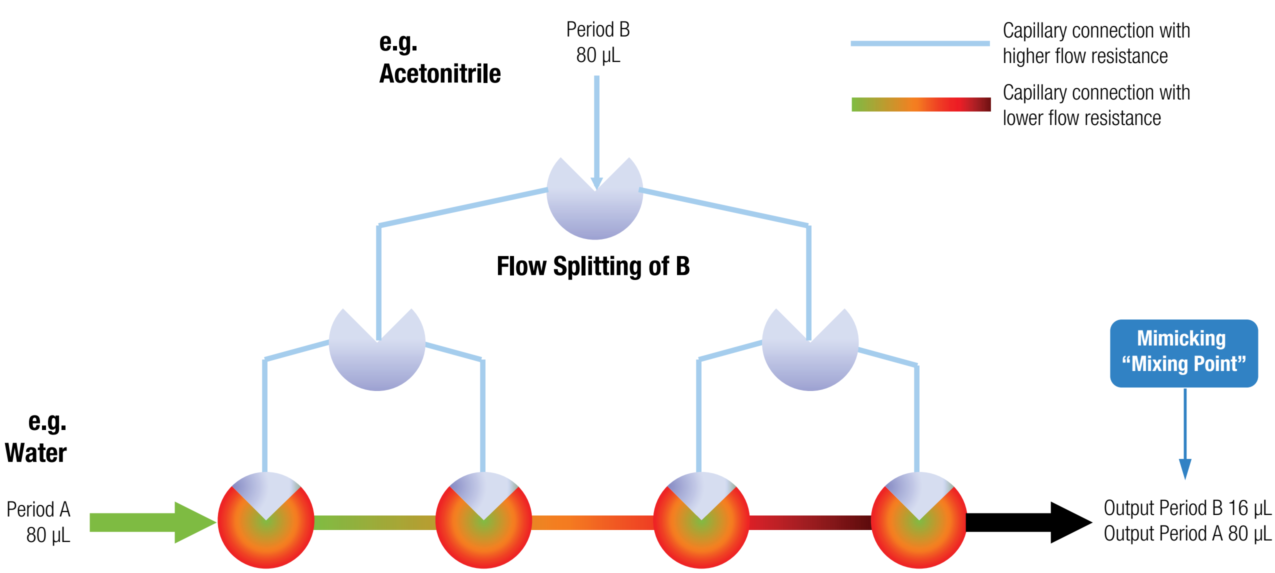


**Figure 4. Simulation of a small stroke volume of the pump.**

## Flow consistency

So far it was described that reoccurring disturbances within the pump flow could not be resolved by using larger mixer volumes. Smaller stroke volumes may improve, but not resolve, the TFA baseline behavior; moreover, smaller stroke volume cannot be set for pump designs that have fixed stroke volumes like the pump of the Vanquish Flex Binary system.

It is important to understand that disturbances of the pump flow can be caused by the thermal constants of the solvents. These effects are less pronounced using water, as it has a high thermal capacity and a low coefficient of expansion, but are generally more significant when using organic solvents, e.g. acetonitrile. These physical properties affect how the solvent volume changes during the pressurization phase in a pump cycle. For water, less compression work must be done compared to that for acetonitrile. From a thermodynamic perspective, water warms up negligibly, whereas acetonitrile heats up notably. After the compression phase of the pump cycle, a time-dependent cool-down to ambient temperature of the compressed solvent takes place in the piston chamber corresponding to a volume reduction, resulting in a flow delivery lower than intended.

Subsequently, the fluctuation of the flow causes the disturbance that is responsible for the variation of the TFA concentration and is amplified by the column. To achieve a low-noise UV trace over the entirety of an applied LC gradient run in TFA applications, disturbances of the flow must be eliminated appropriately (Figure 6).

Smart pump control algorithms are able to compensate this negative flow portion and thus provide a more consistent and accurate flow. This results in a much more even UV baseline with only minimal residual ripples and without the need of major mixer volume adjustments that negatively impact the analysis time. Figure 6 impressively illustrates how substantial the impact of enhanced flow control is on the UV baseline behavior. The left graph displays a TFA baseline for an established UltiMate 3000 Binary RS system with a high level of baseline ripples, which may interfere with analyte peaks, thus reducing reproducible peak integration. The right graph illustrates the superior pump control technology of the Vanquish Flex Binary system, revealing a massive improvement in the TFA baseline noise (Figure 6).

## Conclusion

The new pump control algorithm enables the Vanquish Flex Binary system to offer both ultra-low baseline ripple and low GDV at the same time. The system does not require a pulse damper, which would increase the system GDV (and pressure-dependent), thus limiting throughput. A variety of mixers can be used to tailor the system to the LOD needs of various applications. Nonetheless mixer volume adjustment can be minimized due to the new pump control algorithm of the Vanquish Flex Binary system.

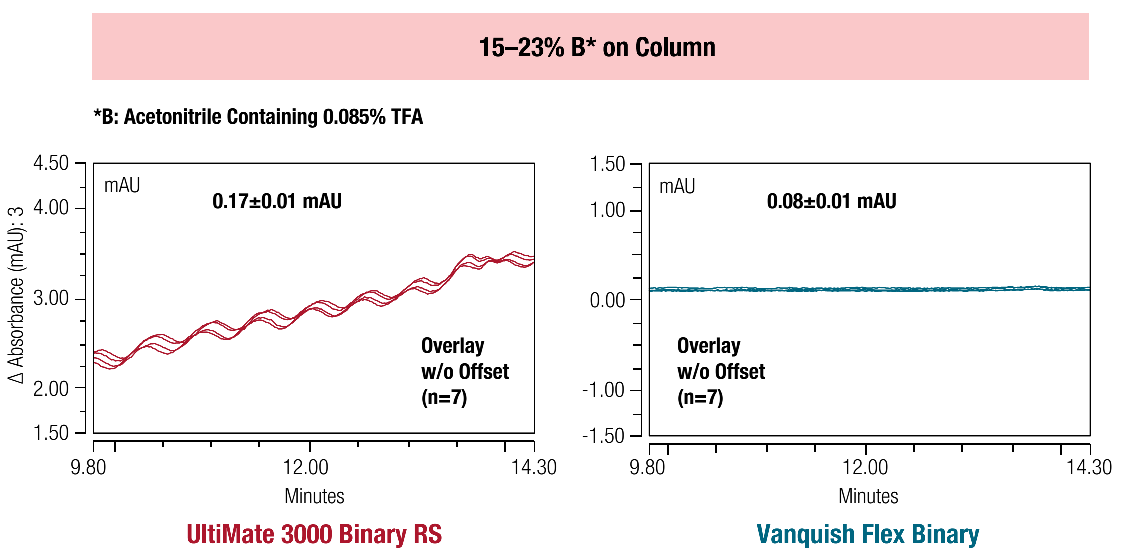


**Figure 6. System comparison of TFA mixing performance.** The UltiMate 3000 Binary RS system and the Vanquish Flex Binary system tested for TFA triggered mixing ripples with the same pump mixer volume (400 µL) in a challenging TFA application (for details, see Figure 2).

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