High-Speed Solid Phase Peptide Synthesis Protocol using PepTip[1] on the Agilent Bravo Automated Liquid Handling Platform

Generation of Soluble Peptide Standards for Mass Spectrometry or Precursors for Microarrays

Application Note

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Abstract

A high-throughput synthesis protocol has been developed implementing the solid-phase peptide synthesis in disposable pipette tips packed with chromatographic material prior producing microarrays or using the synthetic peptides for mass spectrometry. In this protocol PepTip tips were used as solid support to perform standard Fmoc-chemistry based synthesis steps. The Agilent Bravo implementation can be performed to synthesize 384 15- to 20-mer peptides in parallel within 24 hours continuous working time. The resulting peptides can be either used in the tip bound to the solid phase, or can be eluted into a well-plate and mixed with a desired buffer for further use.

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Introduction

Solid-phase peptide synthesis (SPPS) has become one of the most widely used technologies beside recombinant production of peptides and proteins. SPPS has three major advantages: 1) fast peptide synthesis in large and pure quantities, 2) use of natural and unnatural building blocks, and 3) facile handling methods. The solid phase synthesis is usually carried out on polymer resins in small or large columns giving certain degree of parallelization (e.g. 48 columns). However, the biggest limitation here is throughput when larger batches of peptide or libraries were needed. This limitation has been solved with creating chromatographic tips for peptide synthesis which are freely scalable regarding parallelization. Different sizes of PepTip tips can be generated which result in obtaining smaller or larger amounts of crude synthetic peptide. The synthetic peptide can be either implemented in bound form for affinity purifications or can be eluted and then used for spotting many identical microarrays. Also they were used as mass spectrometry standards. As a result these peptides can be applied to highly specific purification protocols when peptides are very crude so that quantitative Multiple Reaction Monitoring (MRM) assays can be generated.

Materials

- Agilent Bravo automated liquid handling platform (9 plates)
- 384-channel LT disposable tip head
- 384 disposable PepTip tips (Glygen Corp.)
- 384-well, polypropylene conical (V-bottom) plate, having 120 mL capacity per well (Axygen)
- Sealing mat (optional)
- Agilent 384 70 mL pipette tips (No 19133-202 or similar)
- Agilent VWorks automation control software
- Amino-acid building blocks, N-terminally protected by FMOC-group and side group protected.
- Solvent for making amino acid solutions: N-Methylpyrrolidone (NMP)
- Wash solution I: N-,N-Dimethylformamide (DMF)
- Wash solution II: absolute ethanol (EtOH)
- Activator I: 25 % N-,N-Diisopropylcarbodiimide (DIC) in NMP
- Activator II: 1-Hydroxybenzotriazol anhydrous (HOBt)
- Fmoc-deprotection solution: 20 % piperidine in DMF
- Side-chain deprotection: 50 % Trifluoroaceticacid (TFA), 45 % Dichloromethane (DCM), 3 % Triisopropylsilane (TIS), 2 % H_2O
- Solubilization solution: 2 % organic base.

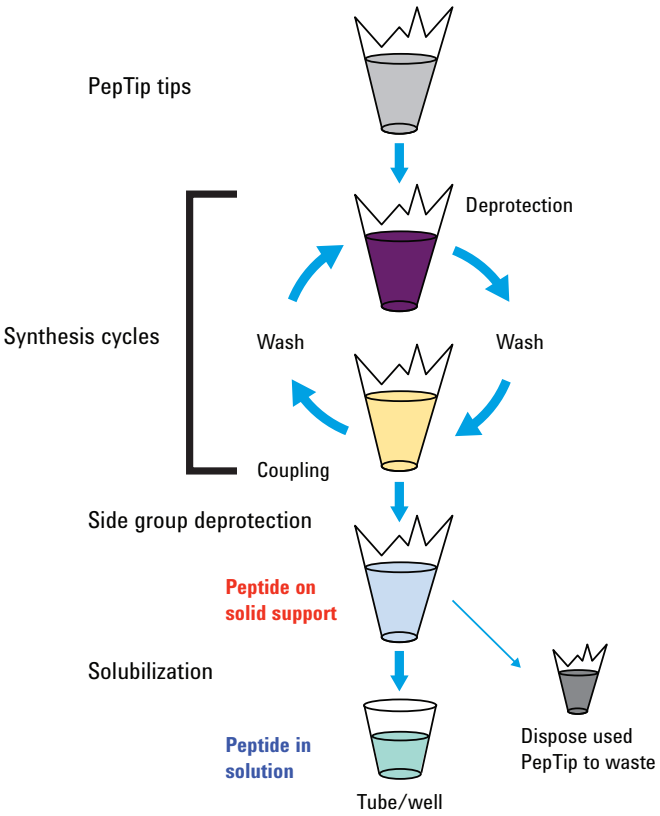

Figure 1: The workflow of peptide synthesis using PepTip tips starting with an amino acid modified PepTip. The main steps are cycles of amino acid coupling and FMOC deprotection, followed by the side group deprotection at the end of synthesis and elution of peptide off the tip. At the end PepTip tips are disposed.

Protocol Workflow

The synthesis protocol described here was implemented as five sequential scripts (A, B, C, D, E and F) written using the VWorks software. The scripts are divided into three groups: "before synthesis" (scripts A and B), "synthesis" (scripts C and D) and "after synthesis" (scripts E and F). Script C and D were repeated in cycles for as many synthesis steps required to couple amino acids to a full length peptide. The plate configuration and labware used are shown in **Figure 2** and listed in **Table 1**.

To prepare for the synthesis **script A** was used to distribute solubilized FMOC-amino acids building blocks into 384-deepwell plates. This script generated the "*coupling plates*", which are needed in script C and D. Each synthesis cycle has its own coupling plate containing 10 mL of well solubilized amino acid in NMP. To place FMOC-protected amino acids building blocks in its target position in a 384-well plate the "hit picking" routine on the Agilent Bravo liquid handler was used. This routine distributed amino acids from a reservoir plate into the target coupling plates (**Table 1, Figure 2a**). Each amino acid was provided in its own 1-2 mL reservoir. The distribution of the individual amino acids into corresponding target wells was driven by a user-defined worklist (in txt- or csv-file format).

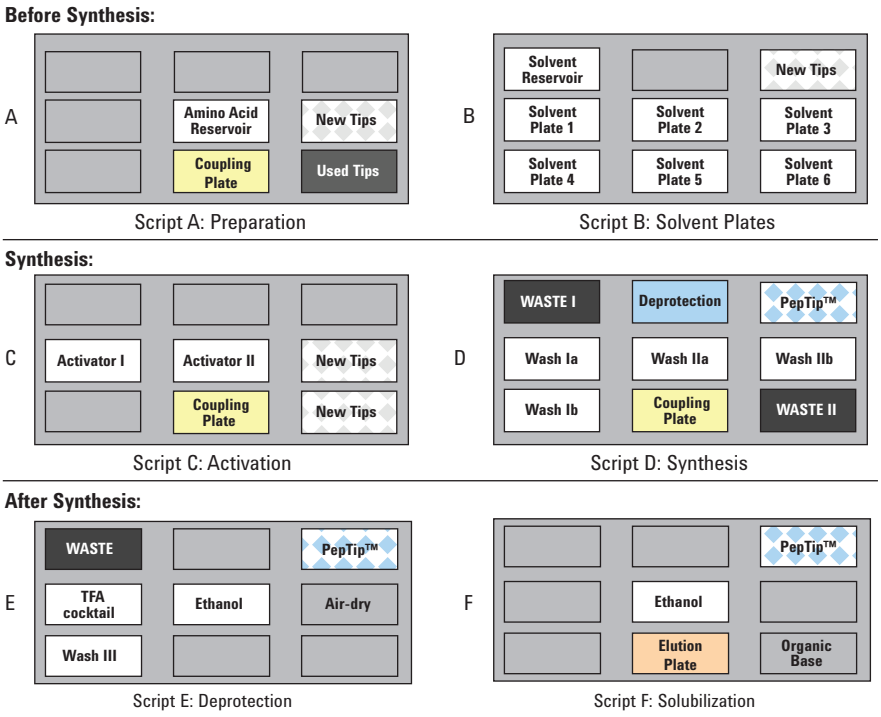

Figure 2: The plate configurations (also layed out in **Table 1**) for all 6 scripts, written in VWorks software. In the following the 6 different configurations are for **A)** distributing the amino acids from a reservoir to target wells in the coupling plate, **B)** generating solvent plates (wash plates and deprotection plates), **C)** activating amino acids in coupling plates, **D)** performing actual synthesis cycles, **E)** removing side group protection from peptides and **F)** solubilizing peptides from solid support.

	Before Synthesis		Synthesis		After Synthesis	
Position	**Script A**	**Script B**	**Script C**	**Script D**	**Script E**	**Script F**
	Preparation:	**Solvent plates:**	**Activation:**	**Coupling:**	**Deprotection:**	**Solubilization:**
1	Empty	Solvent reservoir	Empty	Waste (empty)	Waste (empty)	Empty
2	Empty	Empty	Empty	Piperidine	Empty	Empty
3	Empty	New 50 mL tips	Empty	PepTip	PepTip	PepTip
4	Empty	Solvent plate (empty)	DIC	Wash 1a	50% TFA	Empty
5	AA reservoir	Solvent plate (empty)	HOBt	Wash 2a	Ethanol	Ethanol
6	New 50 mL tips	Solvent plate (empty)	New 50 mL tips	Wash 2b	Empty	Empty
7	Empty	Solvent plate (empty)	Empty	Wash 1b	DCM	Empty
8	Coupling plate (Target)	Solvent plate (empty)	Coupling plate	Coupling plate	Empty	Elution plate (empty)
9	Old 50 mL tips	Solvent plate (empty)	New 50 mL tips	Waste (empty)	Empty	Organic Base

Table 1: Plate configuration of Agilent Bravo liquid handler

The length of the longest peptide determines the number of coupling plates needed to be generated. E.g. a 15-mer peptide needs 15 coupling plates. However, more commonly PepTip tips are sold with the first amino acid covalently attached, hence only 14 coupling plates would be needed in to generate 15-mer peptides. Ideally, each coupling plate has been made in advance in order to go through synthesis steps without interruption. Each peptide was getting one specific well assigned throughout all coupling plates, so that each coupling plate carries the corresponding amino acid of a specific peptide always in the same well position and is sequentially attached to the solid surface in the PepTip tip. The synthesis is performed from the C-terminus to the N-terminus.

Second, after calculating how many wash plates were needed for all synthesis cycles (i.e. 4 wash plates/ cycle and 1 piperidine plate/cycle), the correct number of solvent plates has been generated using **script B**. Solvents DMF or 20 % piperidine were provided in larger 50-100 mL reservoirs and were distributed into all wells of 6 384-deepwell plates at a time (**Table 1, Figure 2b**). For both, DMF and piperidine, only one set of 384 50 µl tips was used throughout the entire **script B**. When first all DMF plates and then piperidine plates were generated, no carryover of piperidine occurred. Solvent plates were removed each time a batch of 6 plates was finished. After completion of all necessary solvent plates, reservoirs and 50 µl tips were removed too.

To start synthesis, the 10 mL of prepared amino acids in each well of the first coupling plate needed to be activated by adding two components (activator I and II) using **script C** (**Table 1, Figure 2c**). For 10 mL solubilized amino acid 5 mL of activator I and 5 mL of activator II was added as follows:

1. Add 5 mL activator I (position 4) in each well of coupling plate (position 8) using new 50 mL tips (position 6).
2. Add 5 mL activator II (position 5) in each well of coupling plate (position 8) using new 50 mL tips (position 9).
3. Mix 10 mL 6 times in wells of coupling plate (position 8) using same tips as in previous step 2.

Once **script C** was finished **script D** had to be run immediately. 5 minutes or less was a good time window between **script C** and **D** to mount new labware. Alternatively to allow quick transition from **script C** to **D** most labware used in **script D** can be mounted already before running **script C**. Labware in **script D** was as follows: One 384 PepTip tip box, 4 previously generated DMF plates, two empty waste plates, one piperidine plate and one coupling plate. The PepTip tip box was placed at position 3, DMF wash plates were put on positions 4, 5, 6 and 7 and empty waste plates were at positions 1 and 9. Piperidine and coupling plate were placed on position 2 (back) and 8 (front), respectively. The layout of the plates is setup such that air-flow (in the fume hood) is from the coupling plate towards piperidine plate and not in opposite direction to avoid contamination by vapor of piperidine. **Script D** (amino-acid coupling) then carried out the following process:

1. Twice deprotection of Fmoc-protection group in PepTip tips over 4 minutes
2. 6 sequential washes of PepTip tips with DMF
3. Three times coupling of new amino acids (position 8) (2 µl each); reaction time 20 minutes per coupling
4. 6 sequential washes of PepTip tips with DMF.

To minimize cross contamination of the coupling solutions with piperidine, two separate wash stations were included in the protocol (one after deprotection (position 4 and 7) the other after coupling (position 5 and 6)). To reduce carry-over PepTips are first 3x washed with 4 µl DMF (position 4 or 5) and then 3x with 10 µl fresh DMF (position 7 or 6), respectively, so that contaminating liquid is not carried into the top of the tips.

After the one synthesis cycle of **Script D** was finished all 384-deepwell plates were removed and new plates mounted for the next step using a new coupling plate dedicated for the next synthesis step. In addition new wash plates, waste plates and one new piperidine plate was mounted too. Alternatively, same piperidine plate can be used, or in case of fixed wash stations and fixed waste containers only the coupling plate needs to be changed. However, it is advised to use especially new wash and waste plates each time to prevent carry-over between synthesis cycles. **Script C** and **script D** were repeated as many times as there were synthesis cycles to be performed, depending on the length of the longest peptide. In the case all peptides have same length all PepTip tips were needed. However, if peptides are of unequal lengths the PepTip tips carrying shorter peptides (optionally) can be removed upon their last amino acid coupling.

One cycle of **Script C** and **D** required about 90 minutes, while the actual liquid handling time was just about 20 minutes. The longest time for the liquid handler was aspirating and dispensing liquid through the PepTip tips for which we chose the option for most viscous liquid class.

Once all synthesis cycles have been completed a final deprotection using piperidine needs to be performed, before moving to **script E**.

Script E was applied only once at the end of the synthesis to free amino acid of their side groups. For this a 384-deep well plate with 120 µl of DCM, a well plate with 120 µl of ethanol and a well plate with 50 % TFA, 50 % DCM was needed. These plates can be produced by using **script A** again, but due to high volatility it is suggested to cover them up with polypropylene sealing caps. Here we worked fast and did not need to cover the plates. All liquid handling is performed in a certified chemical fume hood. **Script E** performed these liquid handling steps:

1. Wash PepTip tips five times with DCM
2. Incubated three times for 1 hour (or 6 x 30 minutes) in 50 % TFA/ 50 % DCM with the use of 10 µl during each aspiration.
3. Wash PepTip tips five times with DCM
4. Wash PepTip tips five times with ethanol

To avoid complete evaporation of DCM and ethanol, these solvent plates were generated immediately before use (using a manual multichannel pipettor). Also in the end the PepTip tips were air-dried (alternatively by applying 5-10 times mixing protocol without any plate in place and just moving air back and forth in the tip. After protocol was completed all 384-deepwell plates were removed.

If the peptides wanted to be obtained in soluble form, script F was applied to elute peptides off the PepTip resin. For this only two plates were needed, one filled with 100 µl organic base in each well (position 9) and one empty plate (position 8). Script F then was running the following process:

1. Incubate PepTip tips for 3-6 hours with organic base, mix intermittently (e.g. every hour)
2. Elute peptides off PepTip tips 4 x with 10 µl fresh organic base

To avoid loss of peptides incubation was done in the elution plate. After obtaining about 50 µl eluate, plates were removed, and PepTip tips were disposed. The elution plate carrying eluted peptides was dried to complete dryness in a speed-vac concentrator. To solubilize peptides again, a desired solvent, e.g. 100 µl water was used. Since solubilization for some hydrophobic peptides might take longer (or even need suitable solvent) it is advised to place the plate on a shaker (with closed lid).

Then soluble peptides were ready for LC-MS analysis or spotting microarrays.

Results and Conclusion

A high-throughput peptide synthesis protocol has been developed using less than 500 µg amino acid to generate 384 individual synthetic peptides with a quantity of about an estimated 1-5 nmol. The entire synthesis protocol is schematically shown in **Figure 1**. The time to synthesize 384 15-mer peptides was under 24 hours of liquid handling. However, since we used a configuration of just one 384-liquid handler arm (instead of e.g. an additional 8-span) we had to manually remove and add new plates every 1.5 hours between each cycle of script D and all other scripts as well. However, this step could be easily replaced with an appropriate robotic arm to manage plates. A series of six separate scripts (A, B, C, D, E and F) were used each with a specific plate layout (**Table 1, Figure 2a-f**). In our configuration the synthesis resulted in soluble peptides ready for further analysis by LC-MS or generating microarrays. Eluted peptides were analyzed by nano LC-MS and nano LC-MS/MS. Examples of resulting chromatographic peaks of selected peptides including mass spectra and tandem mass spectra of selected chromatographic peaks are shown in **Figure 3a-f**. All spectra were generated on an LTQ ion trap (ThermoFisher Scientific, San Jose, CA) hyphenated to a Proxeon EASY-nLC chromatographic system.

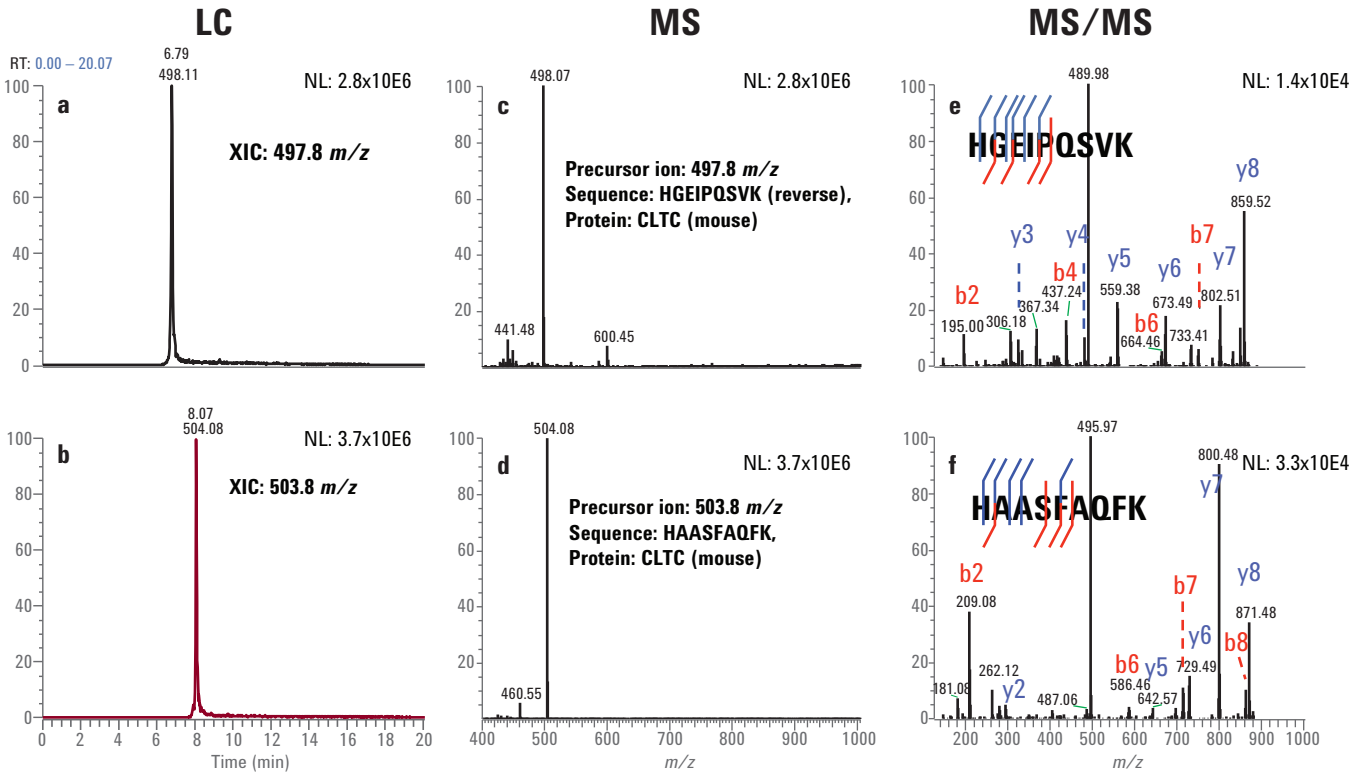

Figure 3: Chromatograms, precursor and product ion mass spectra of two synthesized peptides of the mouse protein CLTC HGEIPQSVK (**a, c** and **e**) and HAASFAQFK (**b, d** and **f**). The first peptide is a reversed sequence of VSQPIEGH with adding a C-terminal lysine (K) after sequence was reversed. With this we show that any peptide not present in nature can be synthesized e.g. for using as false positives. Identified product ion peaks are assigned in **e)** and **f)**. All spectra were generated on an LTQ ion trap (ThermoFisher Scientific).

In about 50 % of cases a clean peptide (>90 % pure) can be obtained and no further purification was necessary. Also, those peptides were directly usable as quantitative peptide standard in mass spectrometry (**Figure 4**) of course after they were quantified by amino acid analysis beforehand.

The clear advantage of this script here is the speed, in which soluble peptides can be obtained within a time of about 5 minutes/peptide from beginning to end. With other configuration of the Agilent Bravo platform it is likely that this number can be further improved. With the growing need of synthetic peptides for microarrays, in-vitro binding studies and MRM-based mass spectrometry, this method can be flexibly applied to all these needs.

Moreover the environmental footprint is extremely minimal since running one entire peptide synthesis protocol of 384 peptides, produced only about 1L of liquid waste (mostly DMF), and another 1L ethanol waste from washing the plates, when they were reused. Due to the individually handled PepTip tips liquid volumes can be optimally adjusted to perform synthesis most efficiently and under the exclusion of water.

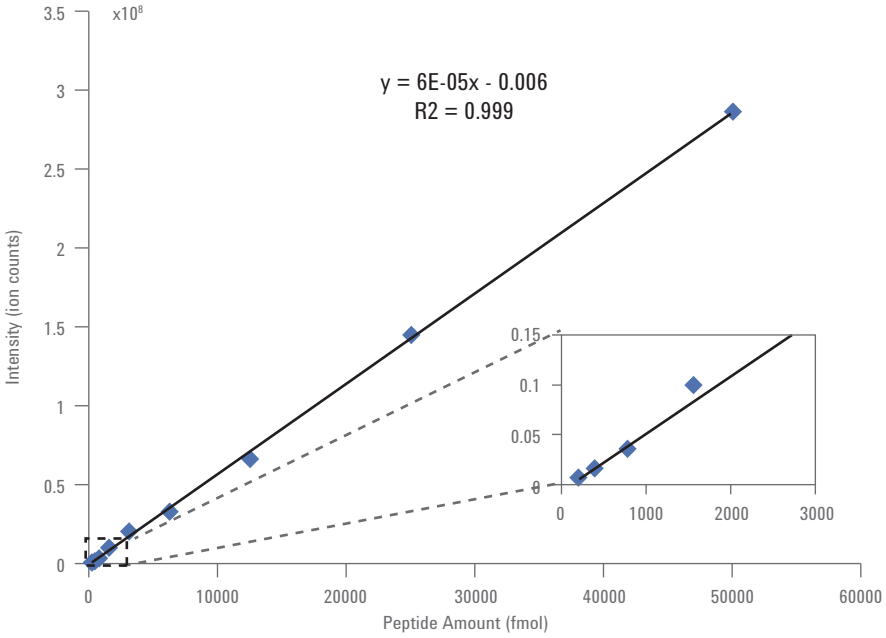

Figure 4: Dilution curve for a synthetic penta-peptide ranging from 50 pmol to about 200 fmol. For quantification peak height of precursor peaks acquired in an Orbitrap mass spectrometer (ThermoFisher Scientific) were used. Zoom in shows the points of lower peptide amounts in the dilution curve. Linearity with R^2 of 0.999 was observed.

1. PepTip tips are a registered US trademark of Glygen Corporation.

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