Solid-phase peptide synthesis in 384-well plates

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SUMMARY

Newer solid-phase peptide synthesis and release strategies enable the production of short peptides with high purity, allowing direct screening for desired bioactivity without prior chromatographic purification. However, the maximum number of peptides that can currently be synthesized per microplate reactor is 96, allowing the parallel synthesis of 384 peptides in modern devices that have space for four microplate reactors. To synthesize larger numbers of peptides, we modified a commercially available peptide synthesizer to enable production of peptides in 384-well plates, which allows synthesis of 1,536 peptides in one run (4 × 384 peptides). We report new hardware components and customized software that allowed for the synthesis of 1,536 short peptides in good quantity (average > 0.5 μmol), at high concentration (average > 10 mM) and decent purity without purification (average > 80%). The high-throughput peptide synthesis, that we developed with peptide drug development in mind, may be widely used for peptide library synthesis and screening, antibody epitope scanning, epitope mimetic development, or protease/kinase substrate screening.

INTRODUCTION

Peptides are attractive for drug development due to their advantageous properties such as the ability to bind difficult protein targets, high target specificity, low inherent toxicity, and ease of development through automated synthesis. Today, more than 80 peptides are used as therapeutics, almost all derived from naturally occurring bioactive peptides, and many are in preclinical and clinical development, including several developed *de novo* by screening random peptide libraries.[1] The efficient development of peptide therapeutics has been enabled by powerful techniques and methodologies introduced over several decades, including solid-phase peptide synthesis (SPPS),[2] automation of synthesis,[3] Fmoc chemistry,[4] reversed-phase HPLC purification, and most recently the development of techniques for the *in vitro* evolution of peptide ligands such as phage display[5] and mRNA display.[6] Over the years, work with peptides has also been greatly facilitated by lowered prices for amino acid building blocks and other reagents and the growing number of commercially available reagents such as hundreds of affordable unnatural Fmoc amino acids.

The development of new peptide therapeutics typically requires the synthesis of a large number of peptides, often in repetitive cycles of peptide synthesis and characterization. The more peptides that can be synthesized and tested in parallel, the greater the chances are of finding variants that exhibit the desired qualities such as potency, selectivity and stability. A peptide development project that nicely illustrates the importance of synthesizing and screening large numbers of peptides in parallel is the engineering of a cyclic peptide ligand of type 2 GPCR glucose-dependent insulinotropic peptide receptor (GIP-R). In this work, a team from Novo Nordisk had established a workflow to identify and prioritize hit peptides from mRNA display selections, synthesized several hundreds of peptides by parallel SPPS in a 96-well format, and ranked them based on activity and other properties. By testing such a large number of peptides, they succeeded in rapidly developing selective GIP-R binders with nanomolar affinity and good stability.[7]

In our laboratory, we have developed methods for generating cyclic peptide ligands *de novo* that are based on synthesizing large libraries of random peptides and screening by functional assays.[8–11] For the production of the libraires, we are using automated SPPS that yields linear peptides that we subsequently cyclize and screen in microwell plates.[10,11] For omitting throughput-limiting purification steps, we have established methods that allow both, deprotecting side chains of peptides on the solid support (so that they can be washed away from the still bound peptides), and selectively releasing the peptides with reagents that do not

interfere with subsequent bioassays. With these methods, we obtained large numbers of peptides at purities approaching or exceeding 90%.[10,11] We further developed methods for diversifying short peptides in combinatorial reactions, for example by acylating 196 short cyclic peptides at an amino acid side chain such as lysine with 100 diverse carboxylic acids, yielding a library of nearly 20,000 cyclic peptide compounds.[9] Screening these libraries in functional assays in 384- and 1,536-well plates led to the identification of nanomolar binders to a range of targets, including thrombin, KLK5, and MDM2.[8,9]

For producing large numbers of peptides with diverse sequences, the synthesis can be performed in parallel in 96-well plates. In our laboratory, we perform peptide synthesis in parallel in four 96-well plate reactors on the MultiPep 2 from CEM/Intavis (384 peptides in one run). Other parallel peptide synthesizers that can synthesize similar numbers of peptides in one run are the Syro I (4 × 96 peptides) and Syro II (6 × 96 peptides) from Biotage, and the Vantage (96 peptides) and Apex 396 (4 × 96 peptides) from Aapptec. SPPS in 384-well format was so far only reported for the synthesis of peptides on membrane discs (4 mm diameter) that were placed into a MultiPep RS 384-well disc-plate holder for synthesis on a MultiPep 2 synthesizer (CEM/Intavis).[12–14] Law and co-workers used this strategy for low-cost peptide microarray generation and mapping of continuous antibody epitopes.[12] Maric and co-workers synthesized peptides on laser-cut membrane disks on the same device and around 30 nmol peptide per well, which was sufficient for protein-protein interaction activity screening.[13] In case the peptides need to be cyclized[8] or diversified in a combinatorial fashion by chemical modification of amino acids side chains for library generation,[11] as anticipated by our laboratory, the quantity of peptide synthesized on membrane disks would likely be too small.

A range of techniques have been developed that allow for the synthesis of peptides in more dense arrays on membranes, wherein the peptides are not released but applied in their membrane-bound form for binding assays similar to immunoblots.[15] Most common applied is SPOT synthesis, where peptides and synthesis reagents are applied in droplets to individual spots on a nitrocellulose membrane and excess is removed by filtration.[16,17] For example, the MultiPep 2 synthesizer together with a 384 CelluSpots membrane holder can produce up to 1,536 peptides that are synthesized in spots of approximately 2–3 mm on four membranes (100 × 150 mm each). Sophisticated techniques were developed to synthesize peptide arrays at higher density, for example based on noncontact inkjet printers that eject pico- to nanoliter volumes of liquid onto a solid support in a predefined pattern[18] and photolithography that uses light to activate selective regions of the solid support for coupling reactions, usually by

removing a photoactive protecting group to allow further synthesis.[19] Release of peptides from membranes to obtain soluble peptide libraries was reported, as for example for developing antibacterial peptides,[20] but the procedure is challenging due to technical hurdles in releasing and transferring peptides from dense arrays to microwell plates, and the much smaller quantities of peptide synthesized on membranes (e.g. ~100 nmol per 7 mm diameter disc) compared to solid-phase resins (e.g. 2 μmol per well in a 96-well plate).

Herein, we aim at synthesizing peptides in 384-well plates using conventional SPPS support such as polystyrene resin (PS) that promised high yields of peptide. To date, no peptide synthesis in 384 well plates on resin was reported and instrumentation for synthesis in 384-well plates is not commercially available. Towards the SPPS in 384-well reactors, we develop hardware parts and adapt software to convert a commercially available 96-well plate parallel peptide synthesizer into a device that can synthesize peptides in 384-well plates and thus 1,536 peptides at once.

MATERIALS AND METHODS

Design and fabrication of adapter frame, and installation of 384-well synthesis plate

Adapter frames were designed with the computer-assisted design (CAD) software Autodesk Inventor Professional 2023. The frames with the dimensions shown in Supplementary Figure 1 were produced by chip removal and utilization of a computerized numerical controlled (CNC) milling machine and polytetrafluoroethylene (PTFE, Teflon) as material (8000943348, APSOparts). Chip removal is a process in which a piece is shaped using tools that can remove excess material. The 384-well plates (PN 201035-100 with PE 25 UM; Agilent) were installed by placing the following hardware parts to one of the four reactor positions of the CEM/Intavis MultiPep 2: 1) standard bottom plate (from 96-well reactor setting), 2) silicon pad (32.402, CEM), 3) newly produced adapter frame, 4) silicon pad (32.402, CEM), 5) PTFE foam pad (32.406, CEM), 6) 384-well synthesis plate, 7) standard top plate (from 96-well reactor setting). The components of the pile were mounted by four new screws with the following dimensions: l = 40 mm, d = 3.9 mm (M4), socket cap screw head: h = 4.0 mm, d = 7.8 mm).

Design, fabrication and installation of 16-channel dispenser

The 16-channel dispenser was designed with the CAD software and produced by chip removal using a CNC milling machine and PTFE (piece a, Supplementary Figure 2), stainless steel (pieces b and c, Supplementary Figure 2), and PEEK (piece d, Supplementary Figure 2; 0253000103, APSOparts) as materials respectively. In addition, 16 needles (0.8 × 40 mm, B. Braun Sterlican) made of stainless steel were prepared. The parts were assembled by inserting the needles into the holes of piece a (depth: middle of 7 mm) and fixing the other parts with four larger (l = 7.3 mm, d = 3.9 mm (M4), head h = 2.5 mm, d = 6.9 mm) and two smaller (l = 7.3 mm, d = 2.9 mm (M3), head h = 2.3 mm, d = 6.0 mm) stainless button head screws. For all six screws, stainless washers were used (four larger: d1 = 9.0 mm, d2 = 4.2 mm, w = 0.75 mm; two smaller: d1 = 6.7 mm, d2 = 3.2 mm, w = 0.5 mm). Each needle is sealed and fixed through the mechanical force applied upon assembly by the underlying channel support. The robotic arm mounting specifications were kept the same as for the standard 8-channel manifold. Optimal dispensing was achieved at a flow rate of 45 ml/min.

Design and construction of top plate for reagent rack

The top plate of the reagent rack was designed with CAD software and produced by chip removal (Supplementary Figure 4). A computerized numerical controlled milling machine and PTFE as material were used. The top plate was assembled outside the peptide synthesizer

with parts of the standard reagent rack of the CEM/Intavis MultiPep 2 by assembling the following parts from bottom to top: 1) new socket head cap screws (l = 30 mm, d = 3.9 mm (M4), head: h = 4.8 mm, d = 7.8 mm), 2) standard bottom plate with openings oriented to the back (from CEM/Intavis MultiPep 2), 3) standard middle plate (from CEM/Intavis MultiPep 2), 4) 5.5 cm standard long spacers (from CEM/Intavis MultiPep 2) with standard grub screws (d = 3.9 mm (M4), l = 20 mm), 5) new top plate, and 6) 2 cm standard small spacers (from CEM/Intavis MultiPep 2). The following tubes can be added to the reagent rack: 4 × 50 ml canonical flat bottom PP tubes (210261, greiner bio-one), 11 × 11 ml round bottom PP tubes (60610, Sarstedt), 7 × 6 ml round bottom PP tubes (38.035, CEM/Intavis), and two of either 48-well (43001-0062, Ritter) or 96-well (260252, Thermo Scientific) PP deep well plates. Optionally, the derivative solutions were covered with a pre-pierced adhesive aluminum lid (Silverseal 676090, greiner bio-one).

Design of resin loader

The basic rectangle dimensions of the resin loader are based on the dimensions of the utilized synthesis plates (Supplementary Figure 2). The volumes of the holes correspond to the volumes of DMF-swollen and filtered resin (aminomethyl PS; 100-150 mesh size, Aapptec) for the desired synthesis scale per well of automated high throughput SPPS. The positions of the holes were chosen so that the resin falls into the wells of the 384-well reactor plate. On all four sites of the loader, edges were added to facilitate aligning of the loader holes to the wells of the reactor place. The edges were designed to contain small gaps allowing facile removal of excess of wet resin during the resin spreading process using a blank PTFE remnant piece.

Definition of instrument modifications in software

The newly designed and produced hardware requires adaptations in the software (configuration file, *.MPC) to allow synthesis in the 384-well plates. The MultiPep software (version 4.4.17) allows custom modification of the reagent rack and reactors. The user manual of the CEM/Intavis MultiPep synthesizer describes how to create and define customized zones for the reagent rack and the reactors to establish customized configuration files. The configuration files are listed in Supplementary Table 4 and the files provided as Supplementary Files.

Setup of modified instrument and calibration of XYZ coordinates

The hardware parts for peptide synthesis in 384-well plates were installed as follows. The 16-channel manifold was mounted to the robot arm by two screws (without the solvent line being connected). The robot arm was then moved to the front right corner of the workspace for connecting the solvent line as follows. The tubing was hold behind the robotic arm and the solvent line was gently screwed into the manifold. This procedure was chosen to ensure that the solvent line was not pinched or dragged by the movements of the robotic arm. The robotic arm was then moved in all dimensions to verify that the tubing was moving freely. The 384-well filter plates were inserted in the following order: top left, bottom left, top right, bottom right (to match order of peptide #). Wells of a 384-well synthesis plate that were not used for peptide synthesis were covered with an adhesive aluminum foil to retain sufficient vacuum pressure for the positions in use. If a synthesis was performed with fewer than four reactor plates, the non-used positions were occupied with 96-well reactor plates that were covered with silicon pads from CEM/Intavis. The modified reagent rack was placed into the synthesizer and all positions filled with empty tubes or deep well plates.

Before each synthesis, the syringe needle and the 16-channel manifold was calibrated so that they were well aligned with the reagent rack and reactor plates. After opening the service software (via "*MultiPep_Service.bat*"), the previously programmed XYZ-coordinates ("X-Pos.", "Y-Pos", "Z-Pos") were found for all the locations available to the software in the "Vials" page ("*Tray Editor*" → "*Vials*"). The bottom toolbar shows the actual XYZ-coordinates of the current position of the needle tip ("actual XYZ:"). At each step described below for a particular position, the previously programmed XYZ-coordinates were compared with the actual XYZ-coordinates observed on manual positioning of the needle tip to the corresponding position. The differences between these two sets of coordinates were then used to "calibrate" the configuration file by changing the previously programmed XYZ-coordinates.

For the reagent rack, the actual XYZ-coordinates were recorded by moving the needle to the XY-center of the tube/well opening and gently moving the needle down until the tip was in contact with the plastic bottom. For tubes, actual coordinates were recorded for the left and rightmost tubes in every row. The average of the XY-coordinate differences observed in these two tubes was then added to all the previously programmed XY-coordinates of that row (which were changed individually via "*Racks*" → **click corresponding rack** → **click blue protractor icon** → *"Vials List (fix)"*; Y-coordinates can be set at once by highlighting all positions and clicking the header "Y"). The Z-coordinate for all tubes in the row was set to the average actual

Z-coordinate observed in the left and rightmost tubes minus a pre-defined offset value (see following table).

For deep well plates, actual coordinates were recorded for the four corner wells of each plate. The average of the XY-coordinate differences observed in these four wells was then added to all the previously programmed XY-coordinates of that plate (which were changed at once via "*Racks"* → **click corresponding rack** → *home position:*). The Z-coordinate for all wells in the microplate was set to the average actual Z-coordinate observed in the four corners minus a pre-defined offset value (Supplementary Table 5). The predefined Z-coordinate offset values are subtracted from the actual plastic bottom coordinate to prevent needle collisions and needle bending. The quality of calibration was checked by commanding the needle to move to some of the reagent rack positions (via *"Vials"* → **click any vial position** → *click green walking man button*). The needle should not contact any solid surface and, when pressed down manually, have 1-2 mm of dead volume between the needle and actual plastic bottom. The Z-offsets recommended for the different tubes are showing in Supplementary Table 5.

For the reactor plates, actual XYZ-coordinates were recorded by moving the needle to the XY-center of the well opening, then gently pressing the needle down until the tip was flush with the opening plane of the well. Actual coordinates were recorded for the four corner wells of each plate. The average of the XY-coordinate differences observed in these four wells was then added to all the previously programmed XY-coordinates of that plate (which were changed at once via "*Racks"* → **click corresponding rack** → "*home position:"*). The Z-coordinate for all wells in the synthesis microplate was set to the average actual Z-coordinate observed in the four corners. The quality of calibration was checked by commanding the needle to move to the middle H12 well of each reactor plate (via *"Vials"* → **click H12 position** → *click green walking man button*). The needle should not contact any solid surface, be nearly perfectly centered in the XY-plane (1 mm tolerance is accepted), and flush with the opening of the well. If a significant off-centering was observed, it should be confirmed that it was not systematic in all four corners of the plate (if so, the calibration was repeated until the centering improved).

For the 16-channel manifold, an accurate dispensing of liquid into the center of reactor plate wells was essential to reduce contamination of the working area with excess piperidine, which could interfere with the following coupling reactions leading to synthesis errors. The manifold

was calibrated simply by initiating a *WashResin* command and then observing the dispensing of liquid droplets (via "*Run Synthesis"* → **click any WashResin task** → **click the green start button**). The liquid droplets should land in the XY-coordinate center of the well of all plates. The experiment was aborted after dispensing to all reactor plates (via *"Pause"* → **click red Abort Run button**). If the droplets were not centered, the approximate correction was added to the previously programmed XY-coordinates of the 16-channel manifold (via *"Tray Editor"* → *"Racks"* → **click any reactor plate** → *"Manifold dX/dY :"*). These changes repositioned the 16-channel manifold independently of the needle. The quality of calibration was checked by restarting any *WashResin* task and again observing the dispensing of liquid droplets. Once the calibration was complete, the experiment was aborted and restarted from the first task to run the actual 384-well plate SPPS synthesis run.

Reagent abbreviations and quality

All reagents were purchased from commercial sources and used with no additional purification. The solvents were not anhydrous nor were they dried prior to use. The following abbreviations are used in this article: Ac_2O (acetic anhydride), MeCN (acetonitrile), BDT (1,4-butanedithiol), DCM (dichloromethane), DIPEA (diisopropyl ethyl amine), 5,5'-dithiobis(2-nitrobenzoic acid) (Ellman's reagent), DMF (dimethyl formamide), DMSO (dimethylsulfoxide), EVA (ethylene-vinyl acetate), FEP (tetrafluorethylen-hexafluorpropylen-copolymer), FFKM (perfluoroelastomer), Fmoc (fluorenylmethoxycarbonyl), HATU (1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium-3-oxide hexafluorophosphate), Mea (2-mercapto-ethylamine), MeOH (methanol), Mpa (3-mercapto-propionic acid), NMM (*N*-methylmorpholine), NMP (*N*-methylpyrrolidone), PE (polyethylene), PEEK, (polyetheretherketone), PP (polypropylene), PS (polystyrene), PTFE (polytetrafluoroethylene), pyridyl-S-S-ethanamine (2-[2-pyridinyldithio]-ethanamine), TEA (triethyl amine), TFA (trifluoro acetic acid), TIS (triisopropyl silane).

Ac_2O (Sigma-Aldrich, >99%), ammonium bicarbonate (Sigma-Aldrich, 99-101%), BDT (Sigma-Aldrich, >97%), MeCN (Fisher Chemical, >99.8%), DCM (Sigma-Aldrich, >99.9%), 2,6-dimethylpyridine (Sigma-Aldrich, >99%), DIPEA (Carl Roth GmbH + Co KG, >99.5%), DMF (Sigma-Aldrich, >99.8%), DMSO (Sigma-Aldrich, >99.5%), Ellman's reagent (Sigma-Aldrich, 99%), Fmoc amino acids and derivatives (GL Biochem Shanghai Ltd, >99%), HATU (GL Biochem Shanghai Ltd, >99%), Mea (abcr, 95%), MeOH (Fisher Scientific, >99.9%), Mpa(Trt) (Combi Blocks, 95%), NMP (Thermo Scientific, 99%), NMM (Sigma-Aldrich, >98%),

piperidine (Acros Organics, 99%), amino methyl PS resin (Aapptec, 100-150 mesh, 1.39 mmol/g), TEA (Fluka Analytical, >98%), water (MilliQ).

Mea-S-S-Mpa-PS resin preparation

The amino methyl PS resin (12.8 g, 17.8 mmol, 1.39 mmol/g) was washed using MeOH (2 × 200 ml), DCM (3 × 200 ml), 1% TFA in DCM (2 × 100 ml), 5% DIPEA in DCM (2 × 100 ml, with incubation for 5 min each), DCM (2 × 200 ml) and DMF (2 × 200 ml) by gently stirring the solvent/resin mixture with a plastic spatula and removing the solvent with a vacuum over a sealed sinter glass filter on a feeding bottle.

The thiol source was coupled to the resin by mixing the pre-washed resin with a pre-activated solution containing Mpa(Trt) (18.6 g, 53.4 mmol, 3.0 equiv.), HATU (20.3 g, 53.4 mmol, 3.0 equiv.) and DIPEA (18.6 ml, 107 mmol, 6.0 equiv.) in DMF (250 ml). The reaction mixture was incubated for 1 h at room temperature and horizontal shaking (200 rpm, IKA KS 260 basic). The resin was washed with DMF (3 × 150 ml). The coupling step and the washing steps were repeated once more before washing with DCM (3 × 150 ml) to facilitate solvent evaporation. The resin was dried over two days at room temperature and under vacuum (~1 mbar). The loading of Mpa(Trt) was determined by weight and amounted to 1.08 mmol/g. Absence of free primary amino groups was confirmed using the ninhydrin test. Potentially remaining amino groups were inactivated by capping using 200 ml capping solution (5% Ac_2O, 6% 2,6-lutidine in DMF) and incubation for 5 min at room temperature. The resin was washed with DMF (3 × 150 ml) and DCM (3 × 150 ml) before continuing with the Trt deprotection.

Deprotecting solution (250 ml, 10% TFA in DCM with 2% TIS) was added and the resin incubated for 1 h at room temperature while horizontally shaking (150 rpm, IKA KS 260 basic). The resin was washed with DCM (3 × 150 ml). The deprotecting and washing steps were repeated once more. The resin was dried overnight under vacuum (~1 mbar) and stored in the freezer at -20°C. For resin stored for more than two months (-20°C), full reduction of the thiol groups was ensured by treating the resin with TCEP. For example for 100 mg of resin (~1.0 mmol/g, 100 μmol, 1.0 equiv.) a solution (1.5 ml) containing TCEP (268 mM, 400 μmol, 4.0 equiv.) and DIPEA (536 mM, 800 μmol 8.0 equiv.) in solvent (NMP/MeOH, 2:1, v/v) was incubated for one hour at room temperature. The TCEP-treated resin was washed with MeOH (5 × 10 ml), NMP (5 × 10 ml), and THF (5 × 10 ml) followed by a qualitative Ellman's reagent test of the washing filtrates.

The disulfide exchange reaction with pyridyl-S-S-ethanamine was performed to introduce the disulfide-linked amino group to the resin. The reaction was performed in 20 ml PP syringes with PE filters (99.278, CEM) and on a 0.8 mmol scale (~0.8 g dry Mpa-functionalized resin, 1.0 mmol/g Mpa-loading) as follows. Per syringe, a solution of 19.5 ml pyridyl-S-S-ethanamine (0.54 g, 2.4 mmol, 3.0 equiv., MeOH/DCM, 3:7; the pyridyl-S-S-ethanamine disulfide was difficult to dissolve but good solubility was achieved by first adding MeOH and then DCM) was added followed by the addition of DIPEA (0.42 ml, 2.4 mmol, 3.0 equiv.). A yellow color appeared upon the addition of the base. The reaction solution was stirred for 3 h at room temperature and rotation (15 rpm, Stuart rotator). The resin was washed with MeOH/DCM (3:7, 2 × 10 ml), DMF (2 × 10 ml), 1.2 M DIPEA in DMF (1 × 10 ml with incubation for 5 min), DMF (3 × 10 ml) and DCM (2 × 10 ml). The DCM wet resin was kept overnight under reduced pressure for drying and stored at -20°C. A qualitative ninhydrin test was performed to ensure the successful introduction of the amino group. An Ellman's test was performed to ensure the absence of thiol groups. As described in the results section, the loading of Mea on the Mea-S-S-Mpa-PS resin was not determined but was assumed to be several fold lower due to the limited yield of the disulfide exchange reaction.

Resin transfer into well plates using a solid dispenser

The resin was filled to synthesis plates using resin dispenser devices, wherein a device with a suitable number of wells (96 or 384) and cavity size was chosen using the size tester. The resin was filled to the cavities of the dispenser in wet form as attempts to apply resin in dry form led to problems due to clump formation. Resin was swollen in a 20 ml syringe (99.278, CEM) containing a PE filter with DMF for 15 min at room temperature. The excess of solvent was discarded and the syringe without piston and cap was centrifuged (216 × g) inside a closed 50 ml canonical tube for 1 min to standardize the DMF content. The wetted resin was poured onto the appropriate solid dispenser. The resin was distributed and excess gently wiped off with another PTFE plate so that all wells were evenly filled. The synthesis plate was inversely placed on top of it, aligned, and fixed by tape. The assembled construct was flipped and centrifuged using suitable rotor buckets with walls (75006449, Thermo Scientific) containing a 10 mm thick ethylene-vinyl acetate (EVA) foam pad (78 263 01, Rayher Hobby GmbH) underneath to protect the synthesis plate's tips during centrifugation. In case centrifugation buckets without supporting walls from Sigma (13421, Sigma) were used, thinner (3 mm) EVA pads were used to ensure that the plate remains in the centrifuge bucket. The resin was transferred from the solid dispenser to the synthesis plate by centrifugationat 900 ×

g (96-well plates) or 1400 × g (384-well plates) respectively. The loading and transfer of two plates took about 5-10 min.

Peptide synthesis with CEM/Intavis MultiPep 2 in 384-well plates

Automated solid-phase peptide synthesis was performed on an Intavis Multipep RSi synthesizer (equivalent to CEM MultiPep 2) using the above-described hardware adaptations. Commercially available 384-well filter plates were used (201035-10 PE 25 UM, Agilent). For each 384-well synthesis plate, around 1.1 g of dry Mea-S-S-Mpa-PS resin was wetted as described above (~3 μmol/well; loading based on Mpa) and loaded into the wells using the solid dispenser. The loading of the resin was 1.08 mmol Mpa per gram resin. As described in the resin preparation section, the loading of Mea on the Mea-S-S-Mpa-PS resin was not determined but was assumed to be several fold lower due to the limited yield of the disulfide exchange reaction. All reactions, reagent transfers and washing steps were performed at room temperature. While we expected that most reactions perform efficiently at room temperature, incubation at higher temperature could be attractive to improve the coupling of difficult building blocks, but for this, a heating device fitting to the 384-well reactors would need to be constructed. All the equivalents of reagents indicated for the peptide synthesis are based on the Mpa loading. The resin in each well was washed with 6 × 70 μl DMF (16-manifold). Coupling was performed with 25 μl of Fmoc amino acid (500 mM, 4.2 equiv.), 27 μl HATU (500 mM, 4.5 equiv.), 6 μl of *N*-methylmorpholine (4 M, 8 equiv.), and 3 μl *N*-methylpyrrolidone. All reagents used were soluble at the applied concentrations. However, for some non-canonical Fmoc amino acids used in later work, solubility issues were observed (not all compound was dissolved, or fully dissolved compound formed precipitate after several days) and concentrations need to be adapted. All components were premixed for 1 min, then added to the appropriate well containing resin and incubated for 45 min without shaking. The final volume of the coupling reaction was 61 μl, and the final concentrations of reagents were 205 mM amino acid, 221 mM HATU, and 393 mM *N*-methylmorpholine. Coupling was performed twice, followed by resin washing with 6 × 70 μl/well of DMF. Fmoc deprotection was performed twice, each time using 35 μl/well of piperidine in DMF (1:4, v/v) for 5 min. After Fmoc deprotection, the resin was washed with 8 × 70 μl DMF. At the end of the peptide synthesis, the resin was washed with 2 × 70 μl/well of DCM using the needle and not the manifold due to dripping caused by the high density of DCM.

Deprotection of peptides in 384-well plates

After DCM washing, the 384-well plates were dried (4 h, room temperature on air) and the protecting groups removed by incubation with 3 × 1 h deprotecting solution (TFA/TIS/ddH_2O, 38:1:1, 40 μl/well). The cocktail was transferred using a multichannel pipette (8 channels, 30-300 μl, VWR) with activated charcoal-protected tips (5469, MBP 200 Solvent Safe ™) while the outlets of the wells were blocked by pressing the tips of the plate onto a 10 mm thick EVA foam pad (76899, Creotime). The plate was pressed with strong force onto the pad wherein the thin well outlets were not damaged, despite the pressure applied. A new and most recommended procedure is by pressing the plates onto pads by centrifugation at 1500 g for 5 min. During incubation, the 384-well synthesis plate was covered with a PP adhesive lid (G070-N, Kisker Biotech GmbH & Co.). While the plates were covered in this work with the adhesive lids, we recently observed that this procedure can lead to cross-contamination. Cross-contamination could be prevented by not covering the plates with lids but leaving them open in a closed chamber. The deprotection solution was removed using a manual plate vacuum filtration station, and the resin was washed with DCM (3 × 60 μl/well) and dried on air for a few hours before continuing with the reductive release. It was important to efficiently remove traces of TFA by fully evaporating DCM, in order to prevent base neutralization in the next step of reductive release. Preferentially, the traces of TFA were allowed to evaporate by leaving the plates over night. Alternatively, traces of TFA may be removed by an additional wahs with DMF.

Reductive release of library peptides and concentration

The 384-well reactor plates containing the deprotected peptides on resin (theoretical: ~3 μmol/well, 1.0 equiv.) were stacked onto 384-well deep well plates (CLS3342, Corning). The peptides were released by applying release solution (30 μl/well, 400 mM TEA and 400 mM BDT, 4.0 equiv. in DMF) using a multichannel pipette (8 channels, 30-300 μl, VWR) with activated charcoal-protected tips (5469, MBP 200 Solvent Safe ™). In later work, larger volumes of release solution (but the same 4.0 equiv. BDT) was found to be more suited (e.g. 60 μl/well, 200 mM TEA and 200 mM BDT, 4.0 equiv. in DMF; also repeated once to obtain a total of 120 μl of eluted peptide). Upon base addition, it was verified that no white smoke developed that would have indicated incomplete TFA removal. The plate stacks were placed into food-grade PP zipper bags (15387154, M-Classic) to avoid the strong smell of BDT during incubation, transport and centrifugation. The plate stacks were incubated overnight at room temperature and centrifuged (485 × g, 1 min, room temperature). The release was repeated once more (5 hours incubation only), the two release filtrates combined, and the combined

filtrates (60 μl/well) acidified with 50% TFA solution in water (7.4 μl/well, 6.5 M, 2.0 equiv. relative to TEA) for avoiding thiol oxidation during concentration. The 384-well deep well plates were sealed using an adhesive aluminum lid (silverseal 676090, greiner bio-one) and pierced with a homemade aluminum 384-well piercer (Supplementary Figure 6) to form 0.3 mm diameter holes into each well to minimize risks of spillovers during rotational vacuum concentration. The solvents were removed using a rotational vacuum concentrator (RVC 2-33 CDplus IR and Alpha 2-4 LSCbasic, Christ) at ~30°C for 5 h and 1750 rpm (24700, Christ plate rotator 1 and 124708 plate buckets) with a gradient of vacuum down to 0.5 mbar within 20 min. The residues formed after the removal of solvent inside each well were dissolved in DMSO (40 μl/well), and the plates were covered with PS lid (greiner bio-one, Easyseal 676001), sonicated, and centrifuged (485 × g, 1 min, room temperature) before determining the concentration using Ellman's reagent.

LC-MS analysis of peptides

Peptide samples were analyzed by LC-MS analysis with a UHPLC and single quadrupole MS system (Shimadzu LCMS-2020) using a C18 reversed phase column (Phenomenex Kinetex 2.1 mm × 50 mm C18 column, 100 Å pore, 2.6 μM particle) and a linear gradient of solvent B (MeCN, 0.05% formic acid) over solvent A (H_2O, 0.05% formic acid) at a flow rate of 1 ml min^{-1}. For all samples, a gradient of 0 to 60% MeCN within 10 min was applied. Absorbance was measured by UV light at 220 nm. Mass analysis was performed in positive ion mode. 100 μl polypropylene (PP) HPLC microvial (Shimadzu, 980-14379) with PP and teflon caps (Shimadzu, 980-18425) were used for all samples. For analyzing peptides after reductive release and final linear peptide stock solutions in DMSO, a 2 μl sample was transferred into 198 μl solvent (MeCN/water/TFA, 50:49.9:0.1%, v/v/v) and analyzed using an injection volume of 2 μl.

Peptide library quantification by absorption using Ellman's reagent

The final linear peptide stock solution concentrations in DMSO were determined by using Ellman's reagent absorption assay in a 384-well plate format. Aqueous 150 mM ammonium bicarbonate buffer (pH 8.0) in 10% DMSO (23.84 μl/well) and 10 mM Ellman's reagent in buffer (6.00 μl/well) were transferred by bulk dispenser (Certus Flex, Fritz Gyger AG) into a 384-well plate (781096, Greiner bio-one) followed by the addition of ~10 mM linear peptide stock solutions in DMSO (135 nl) by acoustic droplet ejection (ECHO 650, Labcyte/Beckman Coulter) amounting to a total volume of 30 μl/well. 384-well PP 2.0 Microplates ECHO

Qualified source plates (PP-0200, Beckman Coulter) were used and transfers were realized with the standard DMSO calibration. The positive control was composed of aqueous 150 mM ammonium bicarbonate buffer at pH 8.0 (23.9 μl/well), 10 mM Ellman's reagent in buffer (6.00 μl/well) and 10 mM TCEP in buffer (90 nl/well). The negative control contained aqueous 150 mM ammonium bicarbonate buffer at (24 μl/well) and 10 mM Ellman's reagent in buffer (6.00 μl/well) only. The assay plate was sealed by an adhesive PS lid (Easyseal, 676001), centrifuged (485 × g, 1 min, room temperature) and analyzed by absorption at 412 nm using a Tecan M200 Pro. The obtained absorption values were compared to a calibration curve established with the same conditions and a purified linear dithiol model peptide. Quantification of the peptide using the Ellman reagent could potentially be confounded by traces of the reducing agent BDT. However, we have repeatedly found that BDT is efficiently and quantitatively removed by the rotary vacuum evaporation method used.

Peptide synthesis with CEM/Intavis MultiPep 2 in 96-well plates

Automated solid-phase peptide synthesis in 96-well plates was performed on an Intavis Multipep RSi synthesizer (4 × 96 well plates; Orochem, OF 1100). Around 0.45 g/plate of dry Mea-S-S-Mpa-PS resin was added to each well of the 96-well plates (loading = 1.08 mmol Mpa per gram resin; the loading of Mea on Mea-S-S-Mpa-PS resin was not determined but assumed to be at several times lower due to the limited efficiency of the disulfide exchange reaction; all the equivalents for the peptide synthesis are based on the Mpa loading). This corresponds to around 5 μmol/well (Mea group). The resin was loaded using a solid dispenser developed for the 96-well plates. The resin in each well was washed with 6 × 225 μl/well DMF (8-manifold). Coupling was performed with 50 μl of Fmoc amino acid (500 mM, 5.0 equiv.), 53 μl HATU (500 mM, 5.3 equiv.), 12.5 μl of *N*-methylmorpholine (4 M, 10 equiv.), and 5 μl *N*-methylpyrrolidone. All components were remixed for 1 min, then added to the appropriate well containing resin for 45 min reaction without shaking. The standard reagent rack (24 × 50 ml canonical and 7 × 11 ml tubes) was used. The final volume of the coupling reaction was 121 μl/well and the final concentrations of reagents were 207 mM Fmoc amino acid, 219 mM HATU and 413 mM *N*-methylmorpholine. Coupling was performed twice followed by resin washing with 6 × 225 μl/well of DMF. Fmoc deprotection was performed twice, each time using 150 μl/well of piperidine in DMF (1:4, v/v) for 5 min. After Fmoc deprotection, the resin was washed with 8 × 225 μl/well DMF. At the end of the peptide synthesis, the resin was washed with 2 × 300 μl/well of DCM. Solvent removal was realized through vacuum filtration, which is integrated inside the synthesis plate holder module.

Deprotection of peptide libraries in 96-well plates

The same deprotecting procedure was applied as for 384-well synthesis plates, except that a larger volume of deprotecting solution (TFA/TIS/ddH_2O, 38:1:1, 300 μl/well) was added with an 8-channel multichannel pipette.

Reductive release of peptide libraries in 96-well plates

The same release and concentration procedure was applied as for 384-well synthesis plates, except that larger volumes of release (50 μl/well, 400 mM TEA and 400 mM BDT, 4.0 equiv. in DMF) and acidification (12.3 μl/well, 6.5 M TFA in water, 2.0 equiv. compared to TEA) solutions were used. The combined release solutions were concentrated in 1.2 ml 96-well PP DWP (260252, Thermo Scientific) using pierced seals (Silverseal 676090, Greiner bio-one). The seals were pierced with a device containing 96 pins (Supplementary Figure 7).

Determination of peptide synthesis duration

The simultaneously created log files from the performed syntheses were evaluated to determine the required time to process the individual peptide synthesis steps (pre-activation and distribution, washing, deprotection etc.) using the CEM/Intavis MultiPep 2 synthesizer in either 96- or 384-well plates together with 31- or 59-reagent reagent racks.

RESULTS AND DISCUSSION

Installation of 384-well synthesis plates

A wide range of 384-well filter plates are commercially available, mainly for protein and DNA filtration applications, but also for oligonucleotide synthesis on solid phase. For peptide synthesis, we chose a type of plate that is made of polypropylene (PP) and contains a polyethylene (PE) membrane. Both of these materials are compatible with the required solvents and reagents, the membrane has pores suitable for DMF retention, and the wells hold volumes of about 100 μl which we considered suitable for a synthesis scale of around 3 μmol, corresponding to ~3 mg of PS resin per well, depending on resin loading. Of four plates evaluated, we found a 384 PP filter plate with a 25 μm pore PE frit that was most suited (PN 201035-100 with PE 25 UM; Agilent). The 384-well plates could not directly be mounted to our CEM/Intavis MultiPep 2 peptide synthesizer using the standard reactor holders because the plate height is 14 mm and thus 17 mm less compared to the 96-well synthesis plates that are suited for the instrument (OF1100, Orochem). In order to compensate for the height difference, we designed and produced adapter frames so that the top of the 384-microwell plates reached the same height as the 96-well plates (Figure 1). We manufactured the adapter frames from the chemical-resistant polytetrafluoroethylene (PTFE) (Supplementary Figure 1). For mounting the plates to the holders, we used stainless screws with shorter heads compared to the standard screws with black plastic heads in order to avoid clashes between the screw heads and the mobile dispensing manifold, that we aimed to have as close as possible to the wells of the 384-well plates for correct liquid dispensing.

Dispensing manifold with 16 channels

The CEM/Intavis MultiPep 2 peptide synthesizer offers a 16-channel manifold for SPOT peptide synthesis. However, the outlets of this manifold are too distant from the installed 384-well plates (18 mm), risking splashing of reagents to neighboring wells. Instead of using the standard manifold, we designed and produced a new manifold that has 16 channels and releases solvents closer to the 384-well plate surface (11 mm closer; distance between tip of channels and 384-well plate surface = 7 mm; Figure 2a). The small distance that hinders spilling to other wells made it possible to use the manifold also for dosing piperidine, a process in which contamination must be avoided because it could lead to major synthesis errors. Using the multichannel manifold instead of a single-channel needle for piperidine dispensing allowed reducing the time needed for Fmoc deprotection around 16-fold, which was important to achieve similar reaction times in the first and last well of the 384-well plates, and shortened the total time needed for peptide synthesis. We built the 16-channel manifold using PTFE and

polyether ether ketone (PEEK) material as housing and 16 non-sharp disposable needles (0.8 × 40 mm; sharp tips were cut off) made of stainless steel (Supplementary Figure 2). The robotic arm mounting specifications were kept the same as for the standard 8-channel manifold. Optimal dispensing was achieved at a flow rate of 45 ml/min.

Reagent racks

Our peptide synthesizer has a standard reagent rack with space for 24 × 50-ml tubes and seven 11-ml tubes (and the same number of pre-activation tubes), which allows the use of 31 different amino acids at maximum. Alternative racks with slightly more tubes are offered commercially too. However, in order to offer space for more unique building blocks needed for the generation of structurally and chemically diverse combinatorial peptide libraries, we constructed a reagent rack that has space for four 50-ml tubes, seven 11-ml tubes (and the same number of 6 ml pre-activation tubes), and a holder for two polypropylene (PP) microwell plates (Figure 2b). In the position of the holders, suitable deep-well plates can be positioned that contain either 48 4.5-ml wells or 96 2-ml wells (Supplementary Figure 3). One of the two plates is used as a reagent holder and one for amino acid pre-activation. We constructed the modified reagent rack using parts of the standard CEM/Intavis reagent rack and a newly designed top plate that we manufactured using the chemical-resistant material PTFE (Supplementary Figure 4).

Software adjustments

The coordinates of the 384-well reactor plates and the modified reagent rack were defined in the MultiPep 2 peptide-synthesis control software version 4.4.17 (Supplementary Configuration Files). Before starting a synthesis, the positions of the reactor plates and the reagent rack are calibrated (see instrument calibration procedure).

Peptide synthesis in 384-well plates

We tested the modified peptide synthesizer configuration by synthesizing 384 short peptides in a 384-well plate reactor. In order to directly test the synthesis of 1,536 peptides in one run, we synthesized the 384 peptides four times, each time in one of four 384-well reactors that were mounted to the modified CEM/Intavis MultiPep 2 synthesizer (Supplementary Table 1). For comparison, we synthesized the same 384 peptides in four 96-well plates using the original peptide synthesizer configuration (Supplementary Table 1). As model peptides, we chose short random sequences that all contain at the N-terminus a 3-mercapto-propionic acid

(Mpa) and at the C-terminus a 2-mercapto-ethylamine (Mea) group (Figure 3a). These terminal thiol-containing groups can efficiently be cyclized by bis-electrophilic reagents for accessing large libraries of macrocyclic compounds (Figure 3b). In each of the four 384-well reactors, we synthesized 96 random peptide sequences that contain two random canonical amino acids (a total of four building blocks per peptide) and 288 random peptides that contain three canonical amino acids (a total of five building blocks per peptide) (Figure 3b). All peptides were synthesized on a PS resin carrying a disulfide-linked Mea group that allows release of peptide by breaking the disulfide bond as described before (Figure 3c).[10] We prepared thiol-functionalized resin by coupling *S*-trityl-3-mercaptopropionic acid (Mpa[Trt]) to aminomethyl PS resin (100-130 mesh; Aapptec) as the texture of this resin could be loaded efficiently into the 384-well synthesis plates with a procedure described in the following. In later experiments, we found that commercially offered thiol-functionalized PS resin (e.g. Polystyrene A SH from Rapp Polymere GmbH, product code HA40004.0, ~0.85 mmol/g and 200-400 mesh) is equally suited, and we recommend the latter one as it can be purchased.

In order to efficiently transfer equal amounts of Mea-S-S-Mpa-PS resin to wells of 384-well plates, we developed resin loading devices that are based on PTFE plates having small conical holes with volumes corresponding to the desired amount of resin, arrayed exactly as the wells of a 384-well plate (Figure 4). The resin was placed onto the device, distributed by spreading with a blank PTFE plate to fill the arrayed holes and to remove the excess of resin. Next, the reactor plate was placed upside-down on top, and the sandwich turned to transfer the resin to the reactor wells by centrifugation as illustrated in the video (Supplementary Video 1). Similar resin loading devices are commercially available for 96-well plates but not for 384-well plates. The optimal amount of resin was assessed using a "size-tester" resin dispenser having holes of different sizes (Figure 4a, left, and Figure 4b). For the 384-well synthesis, we used resin for a 3 μmol-scale synthesis (~3 mg resin per well; Figure 4a, middle), and for the 96-well reference synthesis, we used resin for a 5 μmol scale synthesis (~5 mg resin per well; Figure 4a, right). We found that the resin loading was more efficient and precise when using wet instead of dry resin. Dry resin was first swollen in DMF in a syringe, the DMF filtered off, the syringe centrifuged at defined conditions to standardize the content of DMF, and spread in wet form over the resin dispenser. We produced the resin dispenser devices using the PTFE as material as this facilitated the transfer of the resin and offered maximal resistance to different solvents (Supplementary Figure 5). Please note that the indicated resin loading (e.g. “3 μmol-scale”) refers to the amount of Mpa on the Mea-S-S-Mpa-PS resin (the Mpa directly linked to the PS resin, not to be confused with the Mpa added to the peptide at the end of the synthesis), and not to the amount of Mea. The amount of disulfide-linked Mea is estimated to

be around 2-3 fold lower than that of Mpa due to the limited yield of the disulfide exchange reaction used to conjugate Mea to the resin. Also, the equivalents of amino acids indicated in the following synthesis refers to the amount of Mpa and not Mea.

For the synthesis in the 384-well format, pre-activated Fmoc amino acids were applied in volumes of 61 μl (205 mM final concentration, around 4-fold molar excess) and coupled twice (45 min each time). The resin was washed with 70 μl volumes of DMF dispensed through the 16-channel manifold. Fmoc deprotection was performed twice, each time adding 35 μl volumes of piperidine in DMF (1/4, v/v) dispensed through the 16-channel manifold. To remove on-resin side-chain protecting groups, we detached the 384-well plates from the synthesizer, sealed the pointed outlet tips by pressing the plates onto a soft ethylene vinyl acetate (EVA) pad, and added around 50 μl TFA solution twice to each well for one hour. To remove the TFA, the EVA pads were removed to allow TFA to drain, and vacuum applied to fully remove TFA. The wells were then washed three times with 52 μl DCM, allowed to dry at room temperature overnight, and the peptides released by disulfide cleavage, adding twice 30 μl DMF containing 1,4-butanedithiol (BDT; 400 mM) and triethylamine (TEA, 400 mM) to each well for one hour. Prior to evaporation of the reagents and solvents, aqueous TFA (6.5 M, 7.4 μl/well; 2 equiv. relative to TEA) was added for acidification, in order to limit dimer formation. The plate was covered applying pierced aluminum seals (homemade puncher, Supplementary Figure 6 and 7) to prevent cross-contamination during the following evaporation process by rotational vacuum concentration. DMF, BDT and TEA were removed by rotational vacuum evaporation. After evaporation, the oily or solid residues were dissolved in DMSO (30 μl/well) to form the desired peptide stock solutions, most of them between 10 to 20 mM. The process of reagent dispensing to 384-well plates is shown in a short video provided (Supplementary Video 2).

Purity and yield of peptides synthesized in 384-well plates

Analysis of ten randomly picked peptides by LC-MS showed that all peptides were correctly synthesized and that they had a good purity (82 ± 6%) (Figure 5a and Table 1). Analysis of the same 10 peptide sequences synthesized in the other three 384-well plates (a total of 30 peptides) showed comparable purities (Supplementary Table 2). The main impurities (0 to 17%, average: 6%) were *t*Bu-capped peptides. Analysis of 40 peptides synthesized in the 96-well plates as a reference (the same 10 peptides in all of the four plates) showed a slightly higher purity (87 ± 6%), with *t*Bu capping impurities ranging from 0 to 16% (average: 5%) (Table 1, Supplementary Figure 8 and Supplementary Table 3). We quantified the peptide

concentrations and yields using Ellman's reagent and measuring absorbance. Peptides synthesized in the 384-well plates had concentrations of 18 ± 5 mM (30 μl elution volume, 3 μmol scale, 18% yield) (Figure 5b) which was comparable to the concentrations found for the 96-well plate synthesis, which was 20 ± 5 mM (50 μl elution volume, 5 μmol scale, 96-well plate, 20% yield) (Supplementary Figure 8). The yields of the synthesis of around 20% appear low but this is because they are calculated relative to the amount of Mpa, and not Mea. The amount of Mea, to which the amino acids are coupled, is several fold lower than that of Mpa due to the low yield of the disulfide exchange reaction. The amount of peptide obtained in the 384-well format was around 0.5 μmol per peptide in average, which is around 15-fold more than obtained by synthesis on membrane disks in a 384-well disc-plate holder (around 30 nmol per 4 mm diameter disc).[13,14] A comparison of time required for the entire liquid handling of the synthesis process showed that 3.3 times less time is required per peptide if they are produced in the 384-well format (without considering synthesizer preparation time; Supplementary Figure 9).

In this work, we tested 384-well synthesis for only relatively short peptides with four or five building blocks (two or three amino acids and the end groups Mpa and Mea) and therefore do not know whether longer peptides can also be efficiently produced. However, in a previous work using the same disulfide linker resin and peptide release strategy in 96-well plates, peptides of about 10 amino acids could be efficiently synthesized,[10] suggesting that peptides in this size range can also be produced in the 384-well format.

CONCLUSIONS

We show that short peptides can efficiently be synthesized on resin in 384-well plates. To establish SPPS in this format, we developed hardware parts and adapted software to modify an existing commercially available 96-well plate parallel peptides synthesizer. With four 384-well plate reactors fitting into the synthesizer, we could produce 1,536 peptides in one run and in less than two days. We also developed a tool for efficiently loading SPPS resin into 384-well reactor plates, and we expanded the number of Fmoc amino acids that can be used to more than 100. The good purity of the peptides, exceeding 80% on average, enables use of the peptides without purification, for example for high-throughput bioactivity screening. The achieved quantity of around 0.5 μmol per peptide in average (30 μl of 10—20 mM peptide stock in DMSO) should allow many applications, ranging from activity screening at high concentrations to combinatorial diversification to generate many variants from each peptide, or potentially even analysis by methods that need larger peptide quantities such as NMR or X-ray co-crystallography.

REFERENCES

1. Muttenthaler M, King GF, Adams DJ, Alewood PF. Trends in Peptide Drug Discovery. *Nat Rev Drug Discov* 2021;20(4):309–325, DOI: 10.1038/s41573-020-00135-8.

2. Merrifield RB. Solid Phase Peptide Synthesis. I. The Synthesis of a Tetrapeptide. *J Am Chem Soc* 1963;85(14):2149–2154, DOI: 10.1021/ja00897a025.

3. Merrifield RB, Stewart JM. Automated Peptide Synthesis. *Nature* 1965;207(4996):522–523, DOI: 10.1038/207522a0.

4. Carpino LA, Han GY. 9-Fluorenylmethoxycarbonyl Amino-Protecting Group. *J Org Chem* 1972;37(22):3404–3409, DOI: 10.1021/jo00795a005.

5. Smith G. Filamentous Fusion Phage: Novel Expression Vectors That Display Cloned Antigens on the Virion Surface. *Science (1979)* 1985;228(4705):1315–1317, DOI: 10.1126/science.4001944.

6. Roberts RW, Szostak JW. RNA-Peptide Fusions for the in Vitro Selection of Peptides and Proteins. *Proceedings of the National Academy of Sciences* 1997;94(23):12297–12302, DOI: 10.1073/pnas.94.23.12297.

7. Bhushan B, Granata D, Kaas CS, Kasimova MA, Ren Q, Cramer CN, White MD, Hansen AMK, Fledelius C, Invernizzi G, Deibler K, Coleman OD, Zhao X, Qu X, Liu H, Zurmühl SS, Kodra JT, Kawamura A, Münzel M. An Integrated Platform Approach Enables Discovery of Potent, Selective and Ligand-Competitive Cyclic Peptides Targeting the GIP Receptor. *Chem Sci* 2022;13(11):3256–3262, DOI: 10.1039/D1SC06844J.

8. Kale SS, Bergeron-Brlek M, Wu Y, Kumar MG, Pham M V., Bortoli J, Vesin J, Kong X-D, Machado JF, Deyle K, Gonschorek P, Turcatti G, Cendron L, Angelini A, Heinis C. Thiol-to-Amine Cyclization Reaction Enables Screening of Large Libraries of Macrocyclic Compounds and the Generation of Sub-Kilodalton Ligands. *Sci Adv* 2019;5(8), DOI: 10.1126/sciadv.aaw2851.

9. Habeshian S, Merz ML, Sangouard G, Mothukuri GK, Schüttel M, Bognár Z, Díaz-Perlas C, Vesin J, Bortoli Chapalay J, Turcatti G, Cendron L, Angelini A, Heinis C. Synthesis and Direct Assay of Large Macrocycle Diversities by Combinatorial Late-Stage Modification at Picomole Scale. *Nat Commun* 2022;13(1):3823, DOI: 10.1038/s41467-022-31428-8.

10. Bognar Z, Mothukuri GK, Nielsen AL, Merz ML, Pânzar PMF, Heinis C. Solid-Phase Peptide Synthesis on Disulfide-Linker Resin Followed by Reductive Release Affords Pure Thiol-Functionalized Peptides. *Org Biomol Chem* 2022;20(29):5699–5703, DOI: 10.1039/D2OB00910B.

11. Habeshian S, Sable GA, Schüttel M, Merz ML, Heinis C. Cyclative Release Strategy to Obtain Pure Cyclic Peptides Directly from the Solid Phase. *ACS Chem Biol* 2022;17(1):181–186, DOI: 10.1021/acschembio.1c00843.

12. McBride R, Head SR, Ordoukhanian P, Law M. Low-Cost Peptide Microarrays for Mapping Continuous Antibody Epitopes. In. 2016; 67–83, DOI: 10.1007/978-1-4939-3037-1_6.

13. Schulte C, Khayenko V, Gupta AJ, Maric HM. Low-Cost Synthesis of Peptide Libraries and Their Use for Binding Studies via Temperature-Related Intensity Change. *STAR Protoc* 2021;2(3):100605, DOI: 10.1016/j.xpro.2021.100605.

14. Schulte C, Khayenko V, Nordblom NF, Tippel F, Peck V, Gupta AJ, Maric HM. High-Throughput Determination of Protein Affinities Using Unmodified Peptide Libraries in Nanomolar Scale. *iScience* 2021;24(1):101898, DOI: 10.1016/j.isci.2020.101898.

15. Szymczak LC, Kuo H-Y, Mrksich M. Peptide Arrays: Development and Application. *Anal Chem* 2018;90(1):266–282, DOI: 10.1021/acs.analchem.7b04380.

16. Frank R. Spot-Synthesis: An Easy Technique for the Positionally Addressable, Parallel Chemical Synthesis on a Membrane Support. *Tetrahedron* 1992;48(42):9217–9232, DOI: 10.1016/S0040-4020(01)85612-X.

17. Hilpert K, Winkler DF, Hancock RE. Peptide Arrays on Cellulose Support: SPOT Synthesis, a Time and Cost Efficient Method for Synthesis of Large Numbers of Peptides in a Parallel and Addressable Fashion. *Nat Protoc* 2007;2(6):1333–1349, DOI: 10.1038/nprot.2007.160.

18. Stadler V, Felgenhauer T, Beyer M, Fernandez S, Leibe K, Güttler S, Gröning M, König K, Torralba G, Hausmann M, Lindenstruth V, Nesterov A, Block I, Pipkorn R, Poustka A, Bischoff FR, Breitling F. Combinatorial Synthesis of Peptide Arrays with a Laser Printer. *Angewandte Chemie International Edition* 2008;47(37):7132–7135, DOI: 10.1002/anie.200801616.

19. Legutki JB, Zhao Z-G, Greving M, Woodbury N, Johnston SA, Stafford P. Scalable High-Density Peptide Arrays for Comprehensive Health Monitoring. *Nat Commun* 2014;5(1):4785, DOI: 10.1038/ncomms5785.

20. Hilpert K, Volkmer-Engert R, Walter T, Hancock REW. High-Throughput Generation of Small Antibacterial Peptides with Improved Activity. *Nat Biotechnol* 2005;23(8):1008–1012, DOI: 10.1038/nbt1113.

ASSOCIATED CONTENT

Supplementary Information is available online

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DECLARATION OF INTERESTS

C.H. and S.H. are co-founders of Orbis Medicines SA. S.H., M.S. and G.S. are employees of Orbis Medicines SA. The other authors declare no potential conflict of interest.

TABLE

Table 1. Comparison of yield and purity for peptides synthesized in 96- and 384-well reactor plates, respectively. The purity was determined by HPLC analysis measuring absorbance at 220 nm. Two of the 10 peptides synthesized in 96-well plates showed a small absorbance and were not included for quantifying the purity. The concentration was quantified by reacting the thiol groups of the peptide with Ellman's reagent and measuring absorbance.

Reactor plate	Scale (μmol/ well)	Number of peptides synthesized	Number of peptides analyzed	Average purity (%)	Concen-tration (mM)	Elution volume (μl)
4 × 96-well plate	5	384	10	87 ± 6	20 ± 5	2 × 50
4 × 384-well plate	3	1,536	10	82 ± 6	18 ± 8	2 × 30

FIGURES

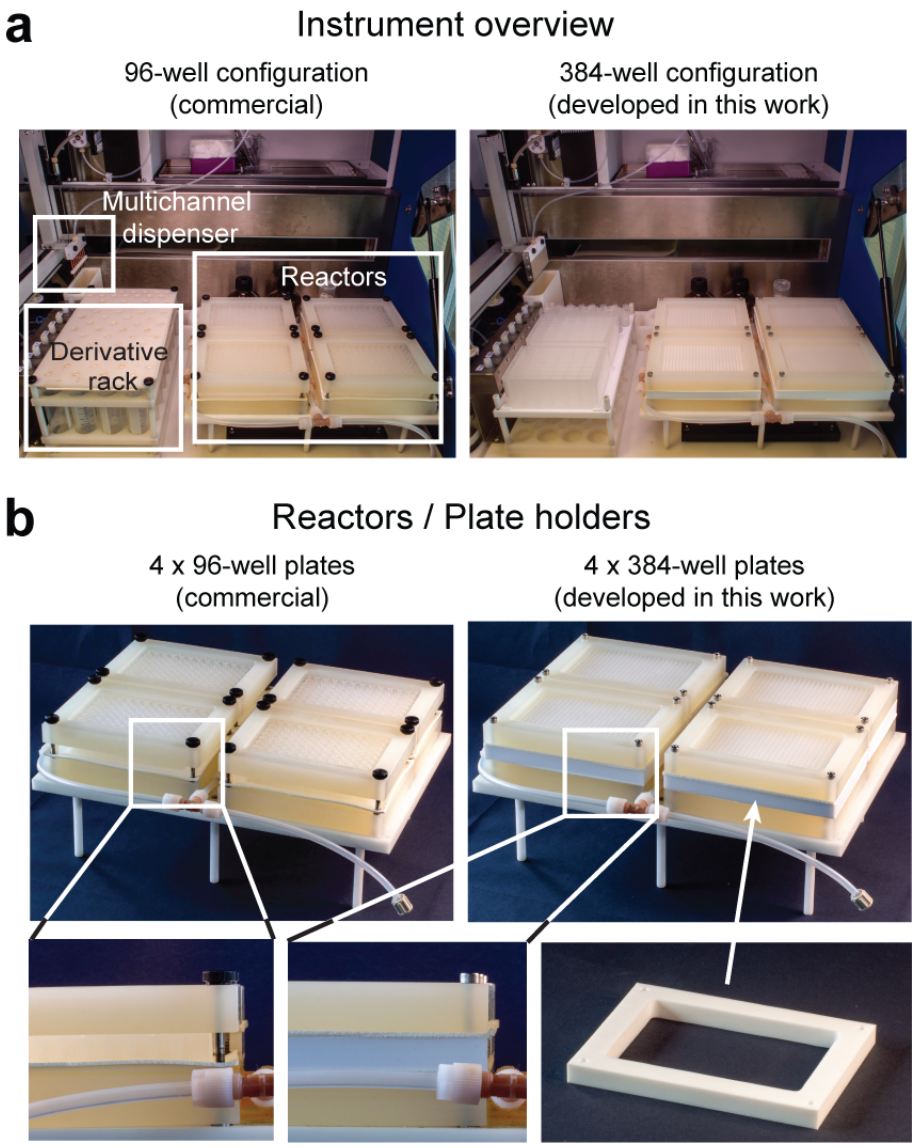


Figure 1. Overview of peptide synthesizer working space and microwell plate reactors. (a) Synthesizer working space. Left: Unmodified peptide synthesizer MultiPep 2 from CEM/Intavis for peptide synthesis in 4 × 96-well plates. Right: Modified instrument for the synthesis in 4 × 384-well plates. (b) Comparison of synthesis reactors. Left: Standard plate holders for 96-well plates. Right: modified plate holders for 384-well plates. An adapter frame (lower right corner) was fabricated to compensate for the smaller height of 384-well synthesis plates. Screws with smaller heads (silver) were used to allow a smaller distance between dispensing devices and reacter plate surface.

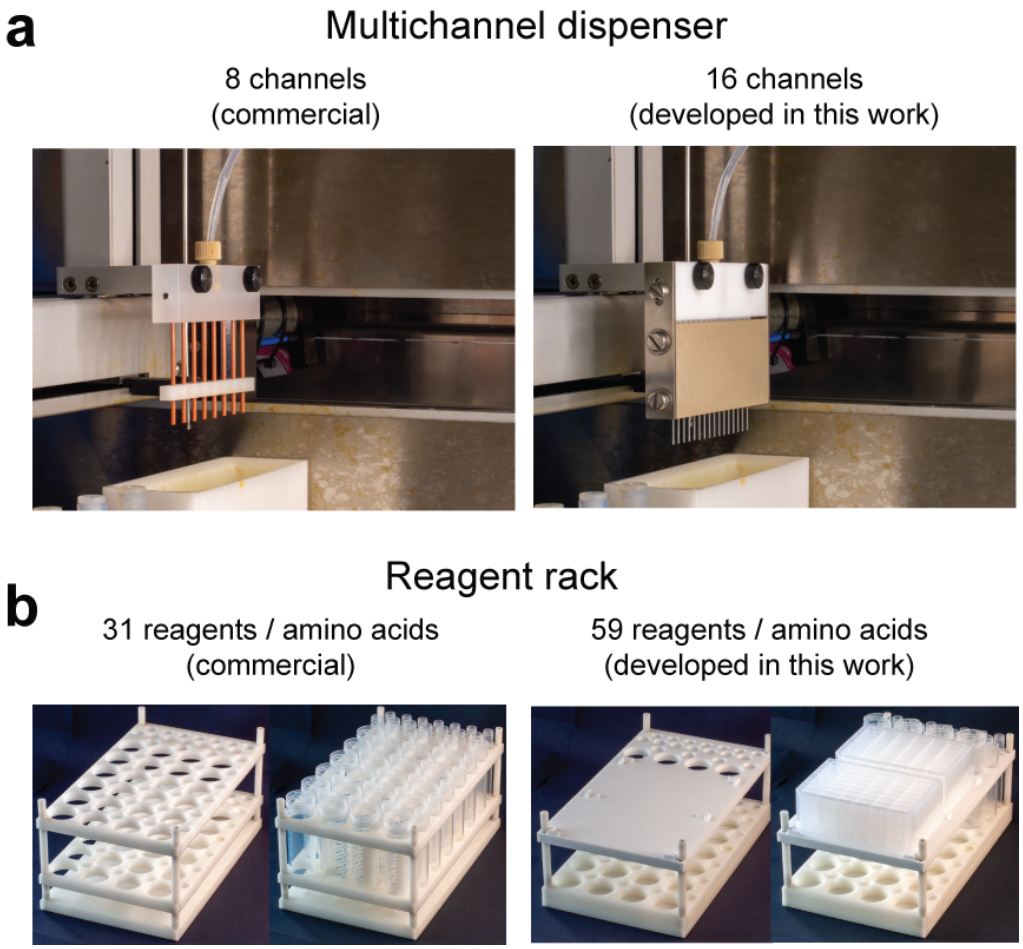


Figure 2. Overview of multichannel dispenser and reagent rack. (a) Comparison of multichannel dispensers. Left: Standard 8-channel dispenser. Right: Custom-made 16-channel dispenser. (b) Comparison of reagent racks. Left: Standard rack for 24 large volumes (50 ml reagent tubes and 11 ml pre-activation tubes) and 7 medium volumes (11 ml reagent tubes and 6 ml pre-activation tubes). Right: Modified reagent rack for 48 or 96 small volumes (e.g. 4 ml volumes in 48-deep well plates), 4 large volumes (50 ml reagent tubes and 11 ml pre-activation tubes) and 7 medium volumes (11 ml reagent tubes and 6 ml pre-activation tubes).

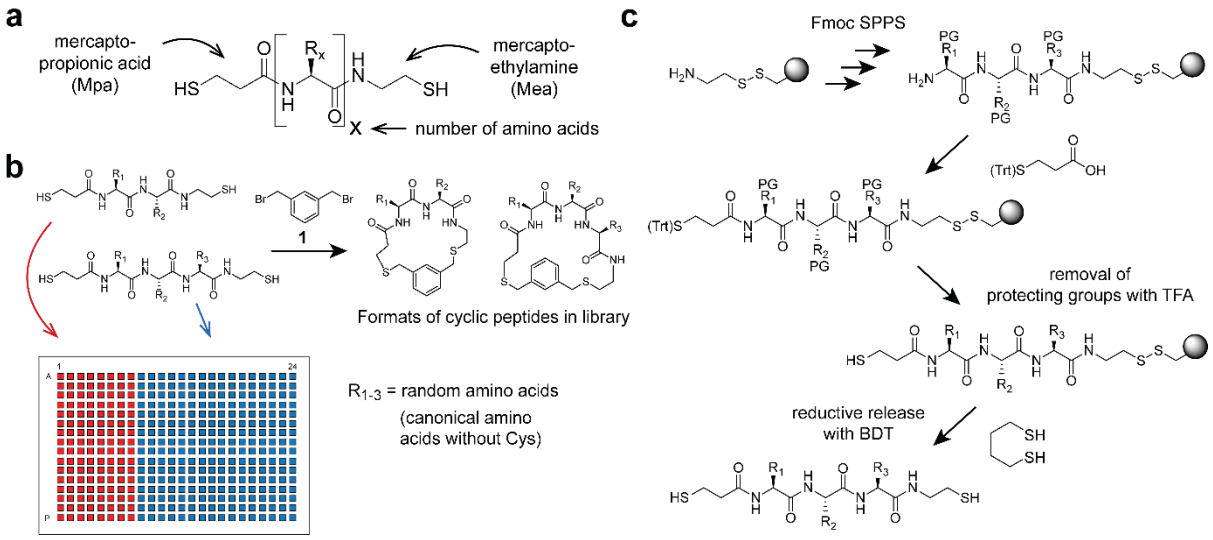


Figure 3. Synthesis of 1,536 peptides in four 384-well plate reactors. (a) Format of the peptides that were synthesized. All peptides contain two or three random canonical amino acids (excluding cysteine) and thiol groups at both ends that allow efficient cyclization by bis-electrophilic reagents. (b) Cyclization of peptides with the bis-electrophilic reagent 1,3-bis(bromomethyl)benzene (**1**). A layout of the 384-well reactor plate with the shorter (red) and longer (blue) peptides indicated is shown. (c) Strategy for the solid-phase synthesis of dithiol peptides. The peptides are synthesized on a disulfide linker resin which allows on-resin amino acid sidechain deprotection and peptide release by reduction.

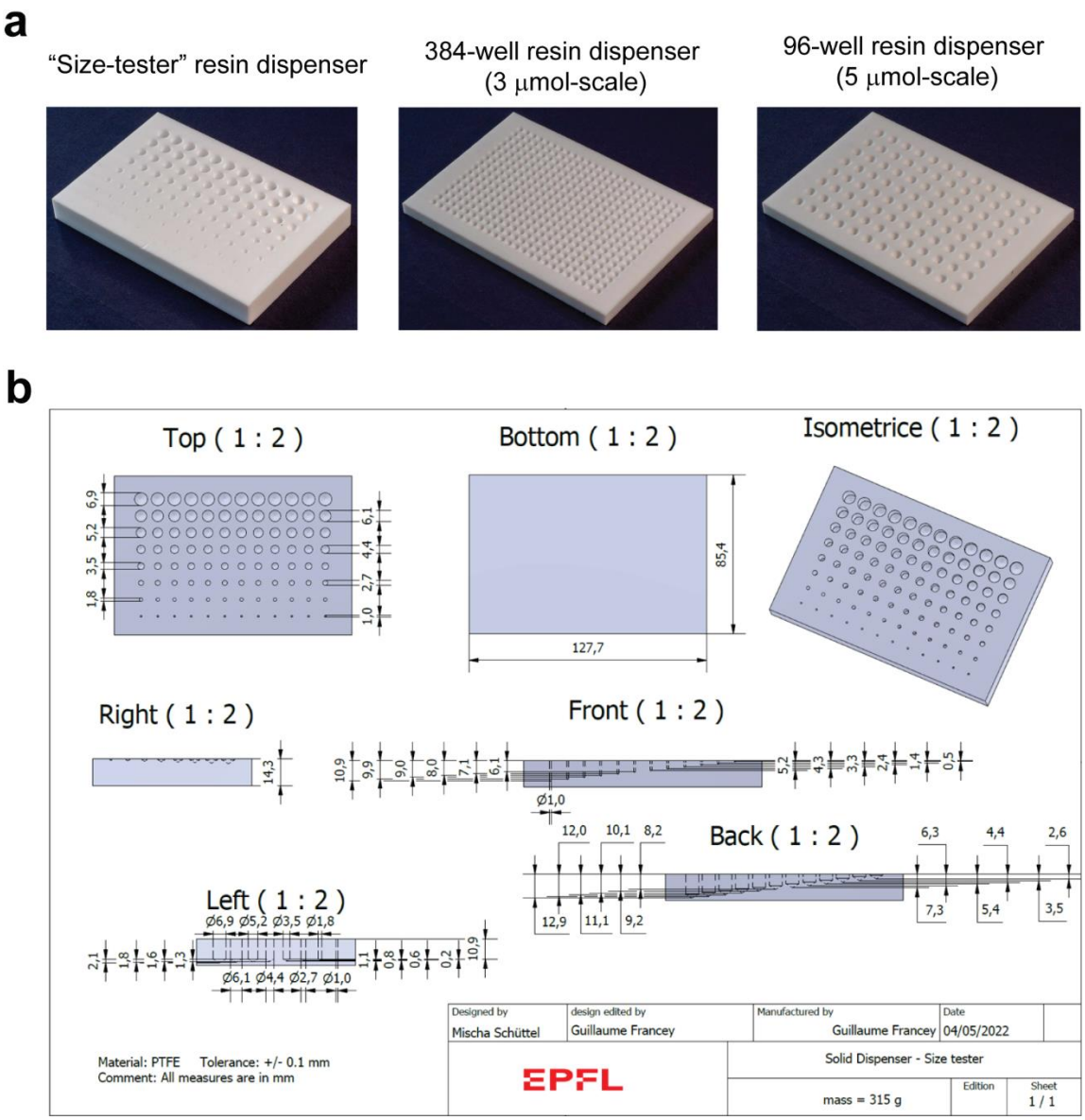


Figure 4. Self-made resin dispensers used for loading of resin into synthesis plates. (a) Size tester (left), 384-well resin dispenser (middle) and 96-well resin dispenser (right). (b) Technical drawing of size-tester.

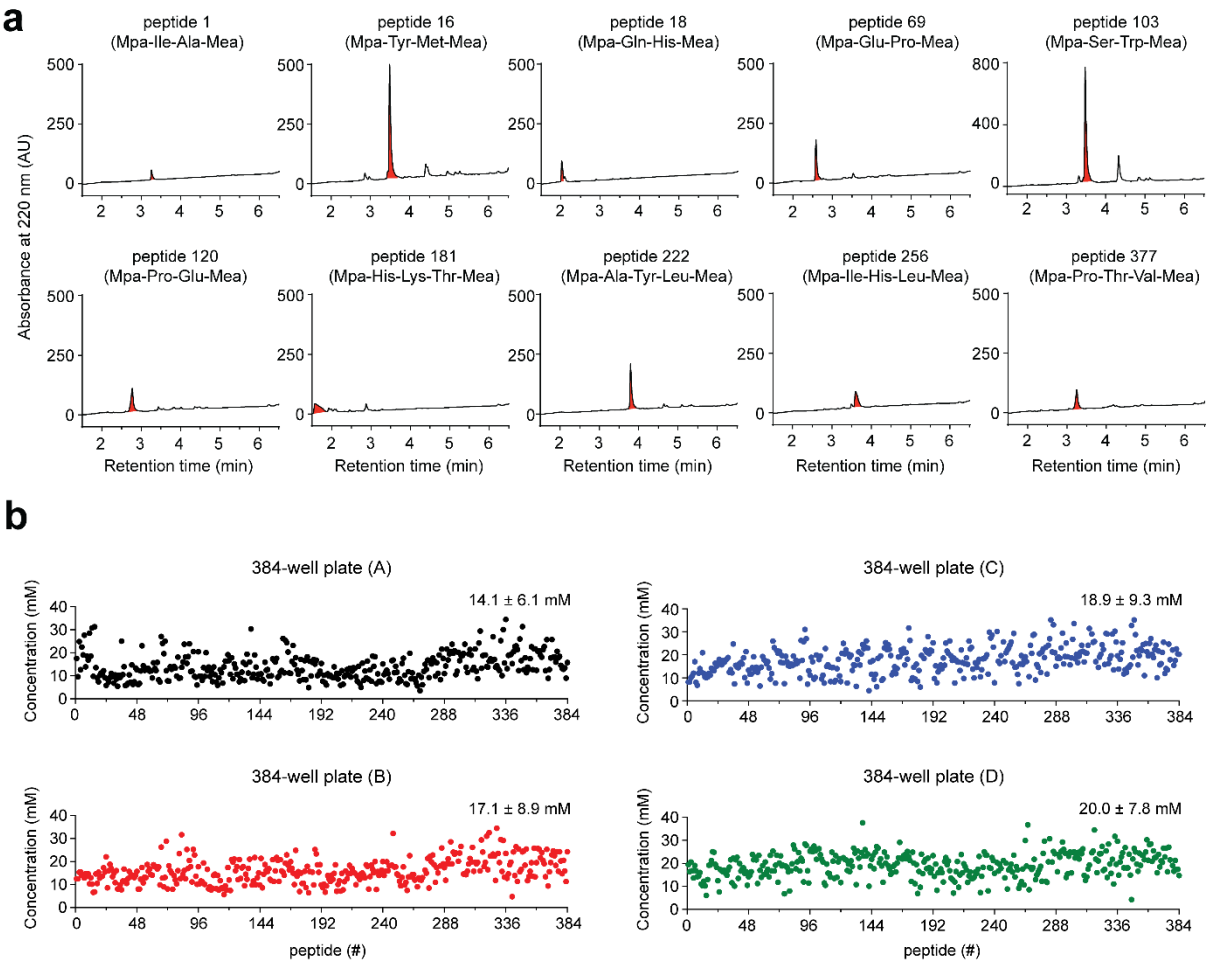


Figure 5. Purity and quantity of peptides synthesized with the modified CEM/Intavis MultiPep 2 in 384-well plates. (a) HPLC chromatograms of 10 randomly picked peptides. (b) Concentrations of peptides quantified by reacting the thiol groups of the peptide with Ellman's reagent and measuring absorbance.

SUPPLEMENTARY INFORMATION

Supplementary Tables

Five supplementary tables showing peptide sequences, peptide LC-MS data, an overview of configuration files, and Z-offset values.

Supplementary Figures

Nine supplementary figures showing technical drawings of hardware parts, a photo of the reagent rack, LC-MS chromatograms, and an overview of the synthesis time.

Supplementary Configuration Files

The following configuration files can be used on a CEM/Intavis MultiPep 2 instrument to define the positions of the 384-well plate reactors and the reagent racks. For the manufacturing of hardware objects presented in this work, 3D files (STEP, STL) are available upon request.

1. MultiPep configuration file for 4 × 96 with 31 reagent rack
2. MultiPep configuration file for 4 × 96 with 59 reagent rack
3. MultiPep configuration file for 4 × 96 with 107 reagent rack
4. MultiPep configuration file for 4 × 384 with 31 reagent rack
5. MultiPep configuration file for 4 × 384 with 59 reagent rack
6. MultiPep configuration file for 4 × 384 with 107 reagent rack

Supplementary Video

The resin loading and peptide synthesis in 384-well plates are shown in Supplementary Videos.

1. Supplementary Video 1: Process of resin loading
2. Supplementary Video 2: Peptide synthesis in 384-well plates

SUPPLEMENTARY INFORMATION

Supplementary Tables

Supplementary Table 1. Sequences of 384 peptides synthesized. EM = exact mass.

ID	coordinate	plate	ID	C-term 5	4	3	2	N-term 1	EM
1	A1	1			MPA	I	A	Mea	349,16
2	B1	1			MPA	S	Q	Mea	380,13
3	C1	1			MPA	N	P	Mea	376,14
4	D1	1			MPA	Y	M	Mea	459,14
5	E1	1			MPA	G	H	Mea	359,12
6	F1	1			MPA	W	R	Mea	507,93
7	G1	1			MPA	S	W	Mea	438,87
8	H1	1			MPA	F	W	Mea	498,90
9	I1	1			MPA	F	I	Mea	425,19
10	J1	1			MPA	S	N	Mea	366,12
11	K1	1			MPA	K	L	Mea	406,22
12	L1	1			MPA	F	K	Mea	440,20
13	M1	1			MPA	Q	D	Mea	408,14
14	N1	1			MPA	M	I	Mea	409,16
15	O1	1			MPA	K	L	Mea	406,22
16	P1	1			MPA	Y	M	Mea	459,14
17	A2	1			MPA	I	L	Mea	391,21
18	B2	1			MPA	Q	H	Mea	430,16
19	C2	1			MPA	Q	Y	Mea	456,16
20	D2	1			MPA	F	D	Mea	427,15
21	E2	1			MPA	Q	F	Mea	440,17
22	F2	1			MPA	T	T	Mea	367,14
23	G2	1			MPA	D	A	Mea	351,12
24	H2	1			MPA	Y	M	Mea	459,14
25	I2	1			MPA	T	E	Mea	395,13
26	J2	1			MPA	W	A	Mea	422,87
27	K2	1			MPA	M	A	Mea	367,12
28	L2	1			MPA	S	N	Mea	366,12
29	M2	1			MPA	M	R	Mea	452,18
30	N2	1			MPA	R	F	Mea	468,21
31	O2	1			MPA	P	F	Mea	409,16
32	P2	1			MPA	T	N	Mea	380,13
33	A3	1			MPA	G	H	Mea	359,12
34	B3	1			MPA	D	Q	Mea	408,14
35	C3	1			MPA	F	I	Mea	425,19
36	D3	1			MPA	H	V	Mea	401,17
37	E3	1			MPA	K	D	Mea	408,18
38	F3	1			MPA	Q	I	Mea	406,18
39	G3	1			MPA	Y	P	Mea	425,16
40	H3	1			MPA	E	K	Mea	422,18
41	I3	1			MPA	F	R	Mea	468,21
42	J3	1			MPA	S	Q	Mea	380,13
43	K3	1			MPA	L	D	Mea	393,17
44	L3	1			MPA	A	S	Mea	323,11
45	M3	1			MPA	H	I	Mea	415,18
46	N3	1			MPA	S	D	Mea	367,12
47	O3	1			MPA	S	F	Mea	399,14
48	P3	1			MPA	P	I	Mea	375,18
97	A7	2			MPA	G	N	Mea	336,10
98	B7	2			MPA	G	M	Mea	353,10
99	C7	2			MPA	T	K	Mea	394,18
100	D7	2			MPA	K	N	Mea	407,18
101	E7	2			MPA	A	K	Mea	364,17
102	F7	2			MPA	N	R	Mea	435,18
103	G7	2			MPA	S	W	Mea	438,87
104	H7	2			MPA	N	W	Mea	465,88
105	I7	2			MPA	K	E	Mea	422,18
106	J7	2			MPA	D	E	Mea	409,13
107	K7	2			MPA	K	D	Mea	408,18
108	L7	2			MPA	I	M	Mea	409,16
109	M7	2			MPA	S	G	Mea	309,09
110	N7	2			MPA	Y	W	Mea	514,90
111	O7	2			MPA	G	T	Mea	323,11
112	P7	2			MPA	V	L	Mea	377,19
113	A8	2			MPA	A	G	Mea	293,10
114	B8	2			MPA	P	D	Mea	377,14
115	C8	2			MPA	Q	N	Mea	407,14
116	D8	2			MPA	S	Y	Mea	415,14
117	E8	2			MPA	K	I	Mea	406,22
118	F8	2			MPA	Y	W	Mea	514,90
119	G8	2			MPA	V	S	Mea	351,14
120	H8	2			MPA	P	E	Mea	391,14
121	I8	2			MPA	S	A	Mea	323,11
122	J8	2			MPA	N	T	Mea	380,13
123	K8	2			MPA	E	E	Mea	423,13
124	L8	2			MPA	L	Y	Mea	441,19
125	M8	2			MPA	G	R	Mea	376,16
126	N8	2			MPA	P	I	Mea	375,18
127	O8	2			MPA	H	W	Mea	488,89
128	P8	2			MPA	G	N	Mea	336,10
129	A9	2		MPA	L	R	F	Mea	581,29
130	B9	2		MPA	P	L	A	Mea	446,21
131	C9	2		MPA	P	P	M	Mea	490,19
132	D9	2		MPA	Q	A	N	Mea	478,18
133	E9	2		MPA	E	A	W	Mea	551,91
134	F9	2		MPA	N	W	I	Mea	578,96
135	G9	2		MPA	Y	F	M	Mea	606,21
136	H9	2		MPA	V	A	R	Mea	491,25
137	I9	2		MPA	D	E	Y	Mea	572,19
138	J9	2		MPA	V	M	P	Mea	492,20
139	K9	2		MPA	R	A	F	Mea	539,25
140	L9	2		MPA	D	L	G	Mea	450,19
141	M9	2		MPA	K	N	W	Mea	593,97
142	N9	2		MPA	V	F	M	Mea	542,22
143	O9	2		MPA	Q	H	T	Mea	531,21
144	P9	2		MPA	N	N	A	Mea	464,16
193	A13	3		MPA	F	R	G	Mea	525,23
194	B13	3		MPA	Q	M	R	Mea	580,24
195	C13	3		MPA	K	N	D	Mea	522,22
196	D13	3		MPA	M	E	E	Mea	554,17
197	E13	3		MPA	Q	Y	D	Mea	571,20
198	F13	3		MPA	F	L	P	Mea	522,25
199	G13	3		MPA	M	W	P	Mea	579,93
200	H13	3		MPA	S	N	I	Mea	479,20
201	I13	3		MPA	Y	N	L	Mea	555,23
202	J13	3		MPA	N	V	A	Mea	449,19
203	K13	3		MPA	S	P	N	Mea	463,17
204	L13	3		MPA	P	A	A	Mea	404,17
205	M13	3		MPA	V	S	G	Mea	408,16
206	N13	3		MPA	G	N	T	Mea	437,15
207	O13	3		MPA	I	R	T	Mea	535,27
208	P13	3		MPA	K	A	L	Mea	477,26
209	A14	3		MPA	Y	F	V	Mea	574,24
210	B14	3		MPA	P	M	M	Mea	524,17
211	C14	3		MPA	P	N	I	Mea	489,22
212	D14	3		MPA	T	H	W	Mea	589,94
213	E14	3		MPA	L	H	A	Mea	486,22
214	F14	3		MPA	G	Q	F	Mea	497,19
215	G14	3		MPA	H	S	Q	Mea	517,19
216	H14	3		MPA	G	S	T	Mea	410,14
217	I14	3		MPA	A	F	Y	Mea	546,21
218	J14	3		MPA	M	S	F	Mea	530,18
219	K14	3		MPA	L	W	M	Mea	595,96
220	L14	3		MPA	D	T	D	Mea	496,16
221	M14	3		MPA	Y	A	G	Mea	456,16
222	N14	3		MPA	A	Y	L	Mea	512,22
223	O14	3		MPA	E	E	I	Mea	536,21
224	P14	3		MPA	L	S	Q	Mea	493,21
225	A15	3		MPA	G	F	S	Mea	456,16
226	B15	3		MPA	T	M	A	Mea	468,17
227	C15	3		MPA	K	Y	Y	Mea	619,26
228	D15	3		MPA	A	M	R	Mea	523,22
229	E15	3		MPA	V	I	N	Mea	491,24
230	F15	3		MPA	R	W	M	Mea	638,97
231	G15	3		MPA	Y	L	T	Mea	542,24
232	H15	3		MPA	Q	G	S	Mea	437,15
233	I15	3		MPA	I	R	K	Mea	562,32
234	J15	3		MPA	L	N	A	Mea	463,20
235	K15	3		MPA	V	F	L	Mea	524,26
236	L15	3		MPA	N	L	K	Mea	520,26
237	M15	3		MPA	G	F	M	Mea	500,17
238	N15	3		MPA	K	Q	N	Mea	535,24
239	O15	3		MPA	N	R	Q	Mea	563,24
240	P15	3		MPA	S	M	M	Mea	514,15
289	A19	4		MPA	P	R	S	Mea	505,23
290	B19	4		MPA	D	I	A	Mea	464,20
291	C19	4		MPA	Q	P	I	Mea	503,24
292	D19	4		MPA	S	Q	F	Mea	527,20
293	E19	4		MPA	S	H	P	Mea	486,18
294	F19	4		MPA	D	V	E	Mea	508,19
295	G19	4		MPA	G	T	N	Mea	437,15
296	H19	4		MPA	D	I	A	Mea	464,20
297	I19	4		MPA	F	W	Q	Mea	626,96
298	J19	4		MPA	Q	R	T	Mea	550,25
299	K19	4		MPA	Y	T	V	Mea	528,22
300	L19	4		MPA	W	W	N	Mea	652,67
301	M19	4		MPA	S	N	G	Mea	423,14
302	N19	4		MPA	R	R	T	Mea	578,29
303	O19	4		MPA	I	K	L	Mea	519,30
304	P19	4		MPA	Q	A	N	Mea	478,18
305	A20	4		MPA	G	E	A	Mea	422,14
306	B20	4		MPA	Q	F	E	Mea	569,21
307	C20	4		MPA	H	H	F	Mea	588,23
308	D20	4		MPA	R	S	W	Mea	594,97
309	E20	4		MPA	L	D	H	Mea	530,23
310	F20	4		MPA	F	L	S	Mea	512,22
311	G20	4		MPA	F	W	L	Mea	611,99
312	H20	4		MPA	V	Q	W	Mea	578,96
313	I20	4		MPA	L	Q	S	Mea	493,21
314	J20	4		MPA	Q	P	S	Mea	477,18
315	K20	4		MPA	R	R	G	Mea	534,26
316	L20	4		MPA	Y	G	N	Mea	499,17
317	M20	4		MPA	F	D	Q	Mea	555,21
318	N20	4		MPA	G	P	A	Mea	390,15
319	O20	4		MPA	I	S	Q	Mea	493,21
320	P20	4		MPA	F	K	P	Mea	537,26
321	A21	4		MPA	E	L	T	Mea	508,21
322	B21	4		MPA	K	N	K	Mea	535,27
323	C21	4		MPA	D	P	Q	Mea	505,19
324	D21	4		MPA	G	I	P	Mea	432,20
325	E21	4		MPA	M	D	H	Mea	548,18
326	F21	4		MPA	M	L	R	Mea	565,27
327	G21	4		MPA	F	M	P	Mea	540,20
328	H21	4		MPA	W	M	T	Mea	583,92
329	I21	4		MPA	W	S	H	Mea	575,92
330	J21	4		MPA	Q	M	P	Mea	521,19
331	K21	4		MPA	T	I	V	Mea	478,24
332	L21	4		MPA	P	E	S	Mea	478,17
333	M21	4		MPA	Q	Q	V	Mea	520,23
334	N21	4		MPA	T	M	R	Mea	553,23
335	O21	4		MPA	W	P	S	Mea	535,92
336	P21	4		MPA	M	I	S	Mea	496,20

Supplementary Table 1. Contiued.

49	A4	1	MPA	L	W	Mea	464,92
50	B4	1	MPA	N	K	Mea	407,18
51	C4	1	MPA	A	S	Mea	323,11
52	D4	1	MPA	V	G	Mea	321,13
53	E4	1	MPA	T	H	Mea	403,15
54	F4	1	MPA	N	P	Mea	376,14
55	G4	1	MPA	L	M	Mea	409,16
56	H4	1	MPA	A	R	Mea	392,18
57	I4	1	MPA	K	Q	Mea	421,19
58	J4	1	MPA	F	R	Mea	468,21
59	K4	1	MPA	Y	N	Mea	442,15
60	L4	1	MPA	E	V	Mea	393,15
61	M4	1	MPA	A	A	Mea	307,11
62	N4	1	MPA	L	R	Mea	434,23
63	O4	1	MPA	A	F	Mea	383,15
64	P4	1	MPA	H	D	Mea	417,14
65	A5	1	MPA	S	N	Mea	366,12
66	B5	1	MPA	R	R	Mea	477,24
67	C5	1	MPA	I	E	Mea	407,17
68	D5	1	MPA	D	Q	Mea	408,14
69	E5	1	MPA	E	P	Mea	391,14
70	F5	1	MPA	P	F	Mea	409,16
71	G5	1	MPA	N	V	Mea	378,15
72	H5	1	MPA	N	H	Mea	416,14
73	I5	1	MPA	Q	F	Mea	440,17
74	J5	1	MPA	G	K	Mea	350,16
75	K5	1	MPA	I	K	Mea	406,22
76	L5	1	MPA	I	V	Mea	377,19
77	M5	1	MPA	G	P	Mea	319,11
78	N5	1	MPA	S	S	Mea	339,10
79	O5	1	MPA	S	D	Mea	367,12
80	P5	1	MPA	T	R	Mea	422,19
81	A6	1	MPA	H	T	Mea	403,15
82	B6	1	MPA	L	T	Mea	379,17
83	C6	1	MPA	P	R	Mea	418,19
84	D6	1	MPA	W	K	Mea	479,93
85	E6	1	MPA	W	I	Mea	464,92
86	F6	1	MPA	Y	W	Mea	514,90
87	G6	1	MPA	H	V	Mea	401,17
88	H6	1	MPA	T	F	Mea	413,16
89	I6	1	MPA	Y	W	Mea	514,90
90	J6	1	MPA	H	A	Mea	373,14
91	K6	1	MPA	G	G	Mea	279,08
92	L6	1	MPA	N	H	Mea	416,14
93	M6	1	MPA	I	P	Mea	375,18
94	N6	1	MPA	I	T	Mea	379,17
95	O6	1	MPA	F	V	Mea	411,18
96	P6	1	MPA	T	K	Mea	394,18

145	A10	2	MPA	Q	N	F	Mea	554,21
146	B10	2	MPA	Y	F	V	Mea	574,24
147	C10	2	MPA	W	R	M	Mea	638,97
148	D10	2	MPA	A	M	E	Mea	496,16
149	E10	2	MPA	S	R	L	Mea	521,26
150	F10	2	MPA	R	H	A	Mea	529,24
151	G10	2	MPA	G	K	A	Mea	421,19
152	H10	2	MPA	M	N	V	Mea	509,19
153	I10	2	MPA	I	M	A	Mea	480,20
154	J10	2	MPA	K	G	K	Mea	478,25
155	K10	2	MPA	H	G	S	Mea	446,15
156	L10	2	MPA	R	I	I	Mea	547,31
157	M10	2	MPA	I	Y	Y	Mea	604,25
158	N10	2	MPA	T	M	Q	Mea	525,19
159	O10	2	MPA	H	S	L	Mea	502,22
160	P10	2	MPA	T	W	E	Mea	581,92
161	A11	2	MPA	W	S	E	Mea	567,91
162	B11	2	MPA	V	H	Y	Mea	564,23
163	C11	2	MPA	T	M	I	Mea	510,21
164	D11	2	MPA	T	M	F	Mea	544,20
165	E11	2	MPA	M	Q	H	Mea	561,20
166	F11	2	MPA	P	H	V	Mea	498,22
167	G11	2	MPA	Y	S	H	Mea	552,19
168	H11	2	MPA	P	W	D	Mea	563,93
169	I11	2	MPA	G	D	G	Mea	394,13
170	J11	2	MPA	Q	E	N	Mea	536,18
171	K11	2	MPA	S	D	K	Mea	495,21
172	L11	2	MPA	Q	A	S	Mea	451,17
173	M11	2	MPA	N	F	K	Mea	554,25
174	N11	2	MPA	K	V	V	Mea	491,27
175	O11	2	MPA	L	K	D	Mea	521,26
176	P11	2	MPA	V	I	D	Mea	492,24
177	A12	2	MPA	H	N	A	Mea	487,18
178	B12	2	MPA	F	I	A	Mea	496,23
179	C12	2	MPA	A	W	S	Mea	509,90
180	D12	2	MPA	M	V	V	Mea	494,22
181	E12	2	MPA	H	K	T	Mea	531,24
182	F12	2	MPA	I	M	I	Mea	522,25
183	G12	2	MPA	P	G	R	Mea	475,22
184	H12	2	MPA	R	D	H	Mea	573,24
185	I12	2	MPA	P	S	K	Mea	477,22
186	J12	2	MPA	G	P	S	Mea	406,15
187	K12	2	MPA	F	R	W	Mea	655,00
188	L12	2	MPA	G	I	M	Mea	466,19
189	M12	2	MPA	A	D	K	Mea	479,22
190	N12	2	MPA	H	E	T	Mea	532,19
191	O12	2	MPA	G	L	S	Mea	422,18
192	P12	2	MPA	K	T	M	Mea	525,22

241	A16	3	MPA	E	G	R	Mea	507,21
242	B16	3	MPA	W	E	F	Mea	627,94
243	C16	3	MPA	Q	M	T	Mea	525,19
244	D16	3	MPA	G	T	K	Mea	451,20
245	E16	3	MPA	S	F	A	Mea	470,18
246	F16	3	MPA	R	A	N	Mea	506,22
247	G16	3	MPA	Q	E	V	Mea	521,21
248	H16	3	MPA	T	S	D	Mea	468,16
249	I16	3	MPA	E	L	Q	Mea	535,23
250	J16	3	MPA	I	G	E	Mea	464,19
251	K16	3	MPA	R	K	I	Mea	562,32
252	L16	3	MPA	M	Q	G	Mea	481,16
253	M16	3	MPA	S	N	L	Mea	479,20
254	N16	3	MPA	H	T	I	Mea	516,23
255	O16	3	MPA	D	Q	F	Mea	555,21
256	P16	3	MPA	I	H	L	Mea	528,27
257	A17	3	MPA	V	P	I	Mea	474,25
258	B17	3	MPA	T	N	W	Mea	566,92
259	C17	3	MPA	F	F	V	Mea	558,25
260	D17	3	MPA	M	G	Q	Mea	481,16
261	E17	3	MPA	M	Y	N	Mea	573,19
262	F17	3	MPA	V	K	W	Mea	579,00
263	G17	3	MPA	A	Y	E	Mea	528,18
264	H17	3	MPA	D	I	R	Mea	549,27
265	I17	3	MPA	K	W	W	Mea	666,72
266	J17	3	MPA	H	R	H	Mea	595,26
267	K17	3	MPA	W	I	L	Mea	578,00
268	L17	3	MPA	G	P	T	Mea	420,16
269	M17	3	MPA	G	I	W	Mea	521,94
270	N17	3	MPA	I	R	D	Mea	549,27
271	O17	3	MPA	P	V	N	Mea	475,20
272	P17	3	MPA	W	M	F	Mea	629,94
273	A18	3	MPA	V	Y	I	Mea	540,26
274	B18	3	MPA	Q	M	P	Mea	521,19
275	C18	3	MPA	S	N	A	Mea	437,15
276	D18	3	MPA	K	N	D	Mea	522,22
277	E18	3	MPA	P	V	N	Mea	475,20
278	F18	3	MPA	H	M	M	Mea	564,18
279	G18	3	MPA	Q	D	M	Mea	539,18
280	H18	3	MPA	N	G	P	Mea	433,16
281	I18	3	MPA	T	F	I	Mea	526,24
282	J18	3	MPA	G	W	I	Mea	521,94
283	K18	3	MPA	H	P	F	Mea	546,22
284	L18	3	MPA	K	T	R	Mea	550,28
285	M18	3	MPA	L	G	V	Mea	434,21
286	N18	3	MPA	Q	V	R	Mea	548,27
287	O18	3	MPA	Y	N	I	Mea	555,23
288	P18	3	MPA	K	N	P	Mea	504,23

337	A22	4	MPA	V	V	A	Mea	434,21
338	B22	4	MPA	Q	D	L	Mea	521,23
339	C22	4	MPA	P	V	V	Mea	460,23
340	D22	4	MPA	G	T	F	Mea	470,18
341	E22	4	MPA	D	K	T	Mea	509,23
342	F22	4	MPA	G	K	K	Mea	478,25
343	G22	4	MPA	L	N	G	Mea	449,19
344	H22	4	MPA	Q	E	K	Mea	550,24
345	I22	4	MPA	F	W	D	Mea	613,94
346	J22	4	MPA	N	G	L	Mea	449,19
347	K22	4	MPA	Y	K	T	Mea	557,25
348	L22	4	MPA	H	G	V	Mea	458,19
349	M22	4	MPA	Q	R	Y	Mea	612,26
350	N22	4	MPA	Q	P	W	Mea	576,94
351	O22	4	MPA	K	R	H	Mea	586,29
352	P22	4	MPA	T	G	T	Mea	424,16
353	A23	4	MPA	D	G	N	Mea	451,15
354	B23	4	MPA	P	E	P	Mea	488,19
355	C23	4	MPA	K	G	Q	Mea	478,22
356	D23	4	MPA	N	F	N	Mea	540,19
357	E23	4	MPA	Y	P	M	Mea	556,20
358	F23	4	MPA	K	M	Q	Mea	552,23
359	G23	4	MPA	L	R	Q	Mea	562,28
360	H23	4	MPA	E	A	R	Mea	521,22
361	I23	4	MPA	S	A	A	Mea	394,15
362	J23	4	MPA	Q	I	T	Mea	507,23
363	K23	4	MPA	H	M	F	Mea	580,21
364	L23	4	MPA	Q	D	L	Mea	521,23
365	M23	4	MPA	D	R	F	Mea	583,25
366	N23	4	MPA	E	W	I	Mea	593,96
367	O23	4	MPA	H	D	Y	Mea	580,21
368	P23	4	MPA	I	Q	M	Mea	537,22
369	A24	4	MPA	T	Q	G	Mea	451,17
370	B24	4	MPA	H	N	G	Mea	473,16
371	C24	4	MPA	V	K	L	Mea	505,29
372	D24	4	MPA	G	Q	V	Mea	449,19
373	E24	4	MPA	G	L	M	Mea	466,19
374	F24	4	MPA	M	R	T	Mea	553,23
375	G24	4	MPA	F	A	I	Mea	496,23
376	H24	4	MPA	F	T	R	Mea	569,26
377	I24	4	MPA	P	T	V	Mea	462,21
378	J24	4	MPA	P	E	R	Mea	547,24
379	K24	4	MPA	G	T	T	Mea	424,16
380	L24	4	MPA	W	L	V	Mea	563,99
381	M24	4	MPA	P	M	Y	Mea	556,20
382	N24	4	MPA	G	Y	D	Mea	500,17
383	O24	4	MPA	E	P	S	Mea	478,17
384	P24	4	MPA	V	Y	R	Mea	583,27

Supplementary Table 2. Analysis of LCMS for peptides synthesized in 384-well plates using the modified CEM/Intavis MultiPep 2 synthesizer. The indicated peptides were randomly picked (the same well in each one of the four 384-well plates). *No peptide found.

Peptide	Plate	Coordinate	Retention time (min)	[M+H]+ (m/z)	Peak area at 220 nm (%)
1	A	A1	3.26	699.1	93
1	B	A1	3.26	699.3	89
1	C	A1	3.26	699.2	89
1	D	A1	3.02*	699.3	93
16	A	P1	3.49	460.1	79
16	B	P1	3.49	460.1	82
16	C	P1	3.48	460.1	83
16	D	P1	3.48	460.1	84
18	A	B2	2.03	431.0	76
18	B	B2	-	-	*
18	C	B2	2.03	431.0	72
18	D	B2	2.03	431.0	73
69	A	E5	2.58	392.0	88
69	B	E5	2.59	392.0	86
69	C	E5	2.58	392.0	88
69	D	E5	2.58	392.0	69
103	A	G7	3.48	439.1	77
103	B	G7	3.48	439.1	76
103	C	G7	3.48	439.1	77
103	D	G7	3.48	439.1	77
120	A	H8	2.77	392.1	85
120	B	H8	2.77	392.1	75
120	C	H8	2.64	392.1	84
120	D	H8	2.77	392.1	91
181	A	E12	1.58	532.2	72
181	B	E12	1.52	532.2	72
181	C	E12	1.64	532.2	83
181	D	E12	1.61	532.2	65
222	A	N14	3.80	513.3	83
222	B	N14	3.79	513.2	79
222	C	N14	3.79	513.2	85
222	D	N14	3.79	513.3	80
256	A	P16	3.60	529.3	80
256	B	P16	3.62	529.3	83
256	C	P16	3.61	529.3	80
256	D	P16	3.58	529.3	81
377	A	I24	3.25	463.1	83
377	B	I24	3.24	463.1	87
377	C	I24	3.25	463.1	90
377	D	I24	3.25	463.2	83

Supplementary Table 3. Analysis of LCMS for peptides synthesized in 96-well plates using the unmodified CEM/Intavis MultiPep 2 synthesizer. The indicated peptides were randomly picked. Peptides 18 and 181 showed no peak (n.d.) in the chromatogram.

Peptide	Plate	Coordinate	Retention time (min)	$[M+H]^+$ (m/z)	Peak area at 220 nm (%)
1	A	A1	3.29	350.1	93.8
16	A	H2	3.5	460.1	80.3
18	A	B3	n.d.	n.d.	n.d.
69	A	E3	2.7	392	89.5
103	B	G1	3.5	439.1	78.1
120	B	H3	2.85	392	92.2
181	B	E11	n.d.	n.d.	n.d.
222	C	F4	3.79	513.3	92.9
256	C	H8	3.64	529.6	86.7
377	D	A12	3.27	463.2	84.8

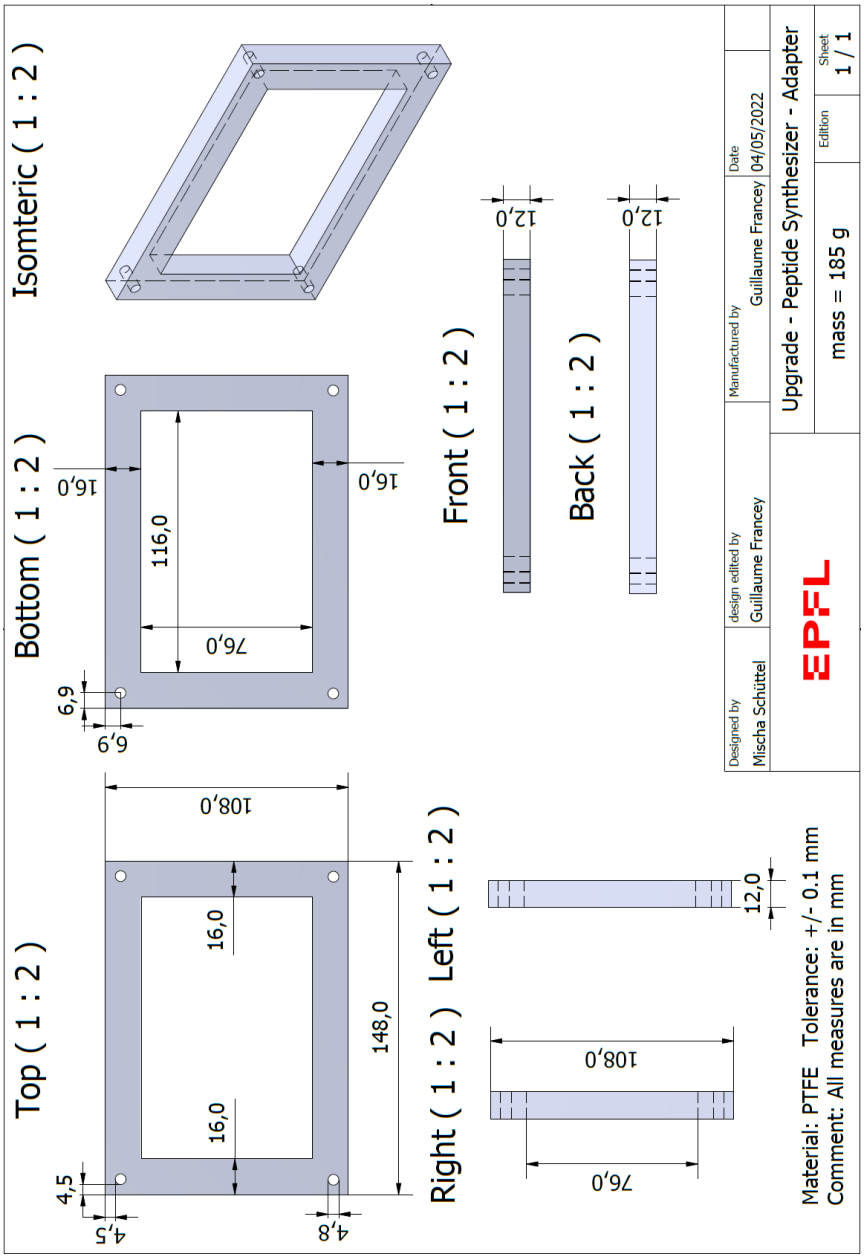
Supplementary Table 4. Overview of customized configuration files.

File name	Reactor	Reagent rack
MultiPepPlates_4x96_31Derivs.MPC	4 × 96-well synthesis plate	31 reagents
MultiPepPlates_4x96_59Derivs.MPC	4 × 96-well synthesis plate	59 reagents
MultiPepPlates_4x96_107Derivs.MPC	4 × 96-well synthesis plate	107 reagents
MultiPepPlates_4x384_31Derivs.MPC	4 × 384-well synthesis plate	31 reagents
MultiPepPlates_4x384_59Derivs.MPC	4 × 384-well synthesis plate	59 reagents
MultiPepPlates_4x384_107Derivs.MPC	4 × 384-well synthesis plate	107 reagents

Supplementary Table 5. Z-offset values recommended for the different tubes.

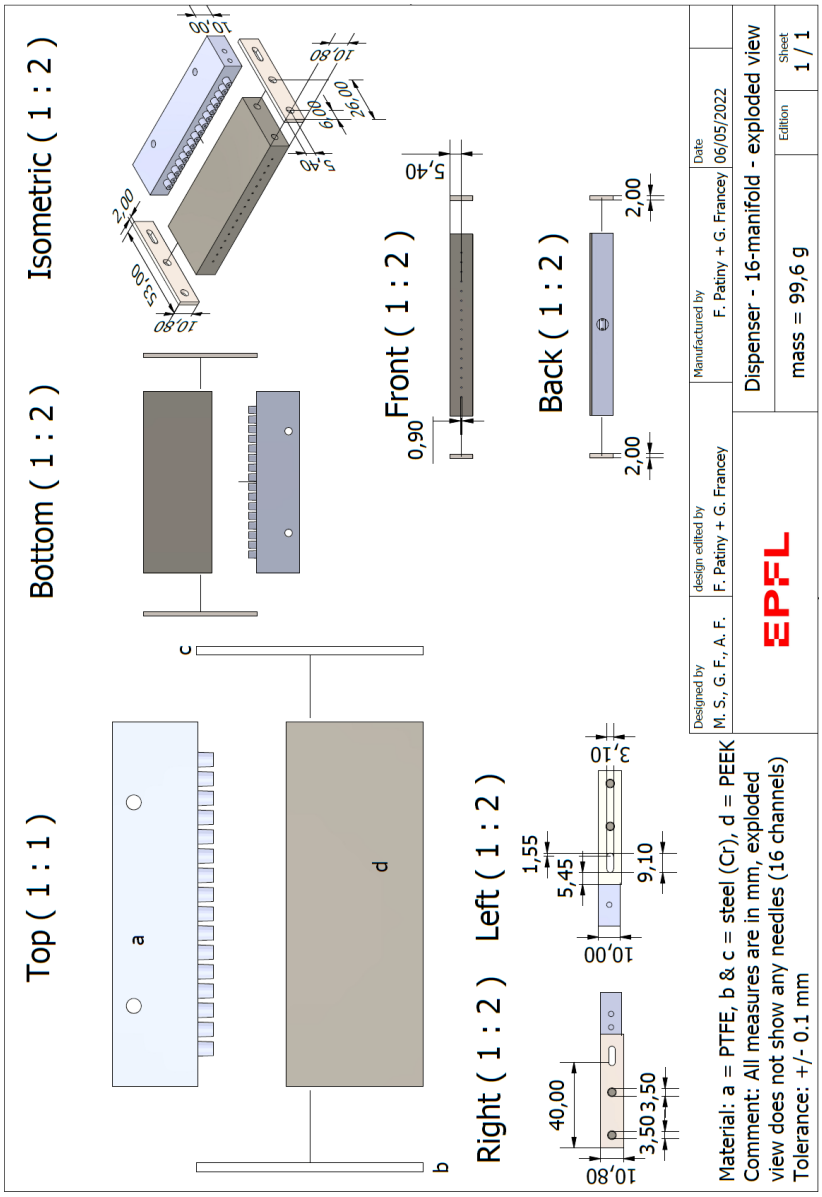
Z-offsets (reagent rack)	
Container	**Z-offset (1 unit = 0.1 mm)**
Small tube (6 ml)	10
Medium tube (11 ml)	10
Large tube (50 ml)	20
48 deep-well plate (4 ml)	15
96 deep-well plate (2 ml)	15

Supplementary Figures



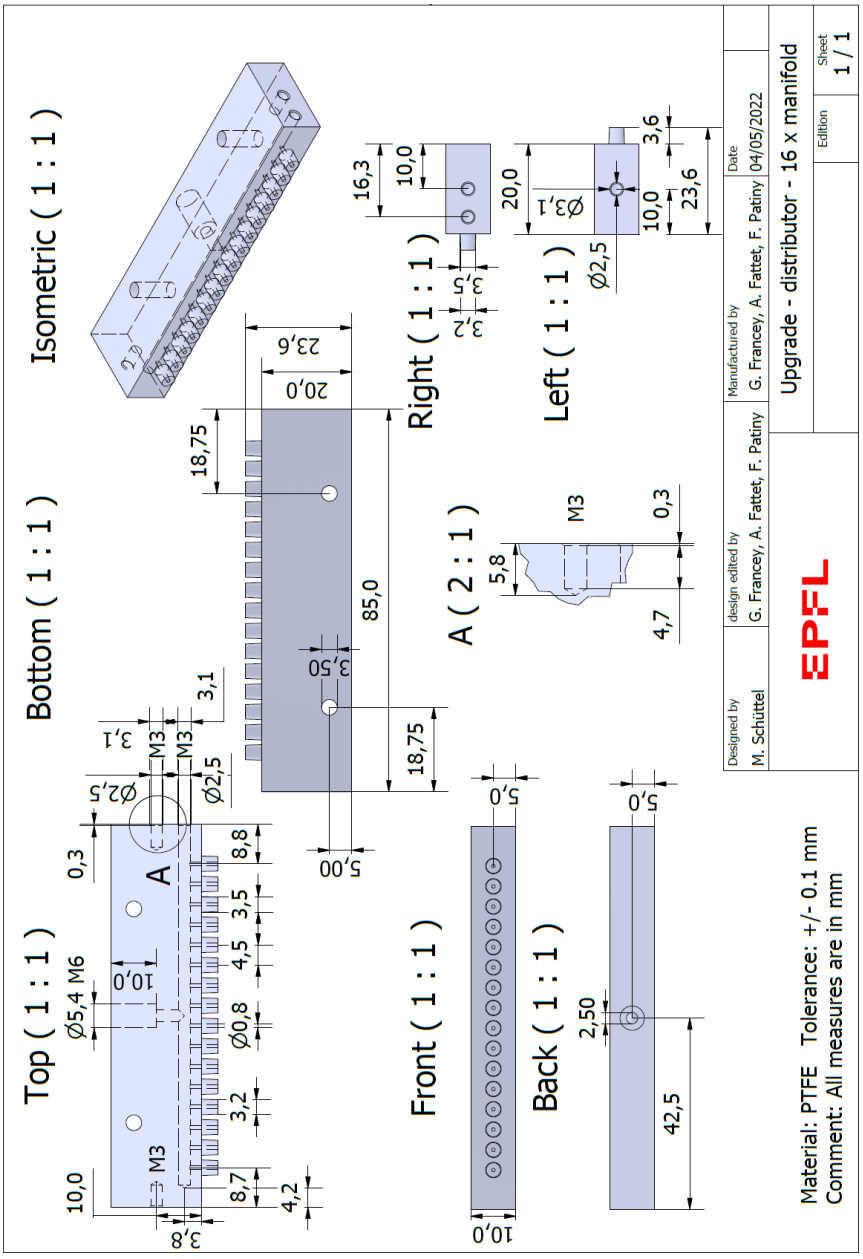
Supplementary Figure 1. Technical drawing of 384-well synthesis plate adapter.

a



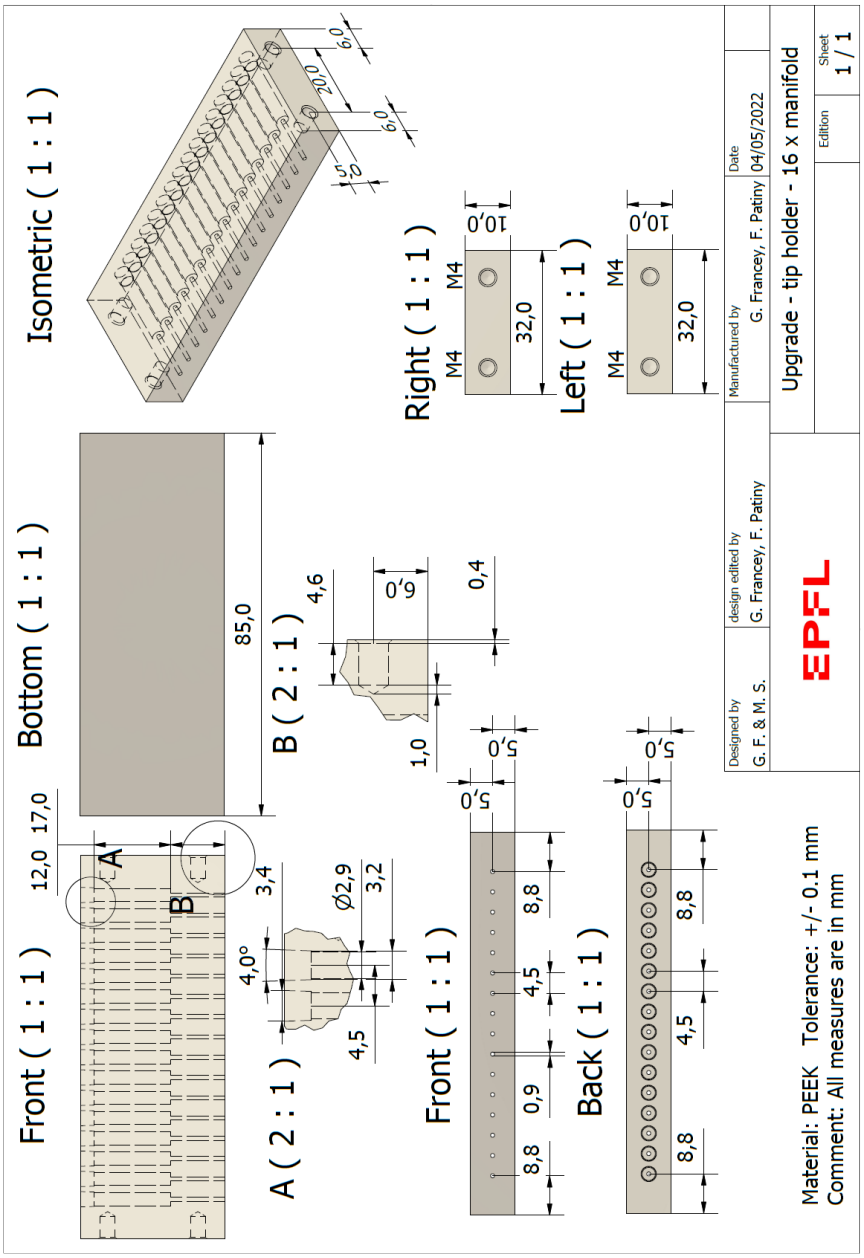
Supplementary Figure 2. Overview of liquid dispensing manifold. (a) Exploded view of the manufactured pieces required for the 16-channel liquid dispensing manifold. The individual blunt needles (Braun, 4657527) for each channel are not visualized. (b) Technical drawing of piece a. (c) Technical drawing of piece d.

b

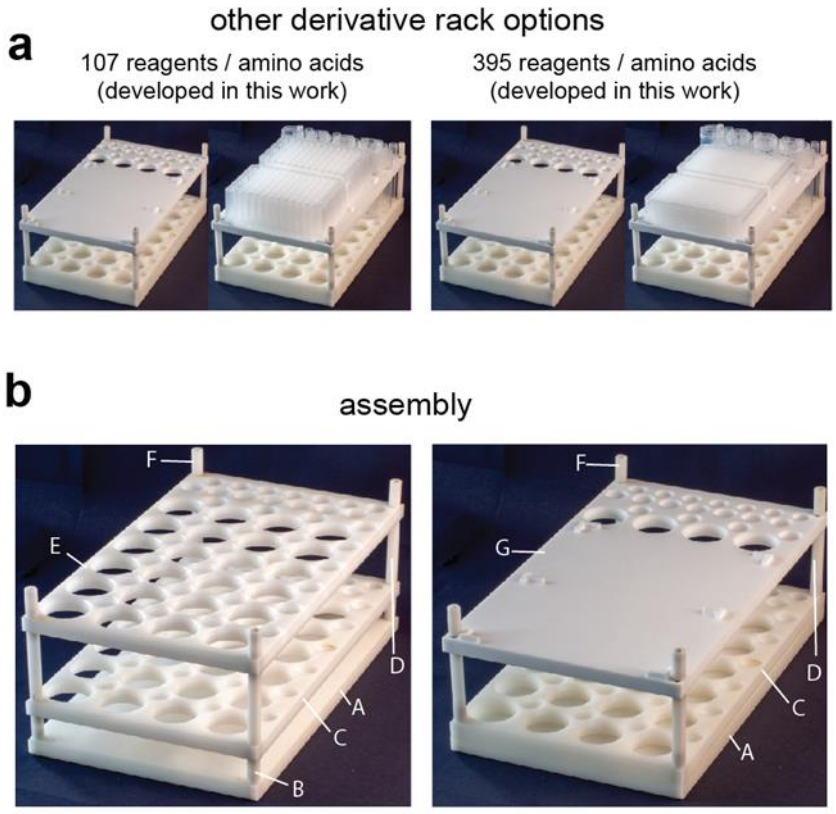


Supplementary Figure 2. Continued

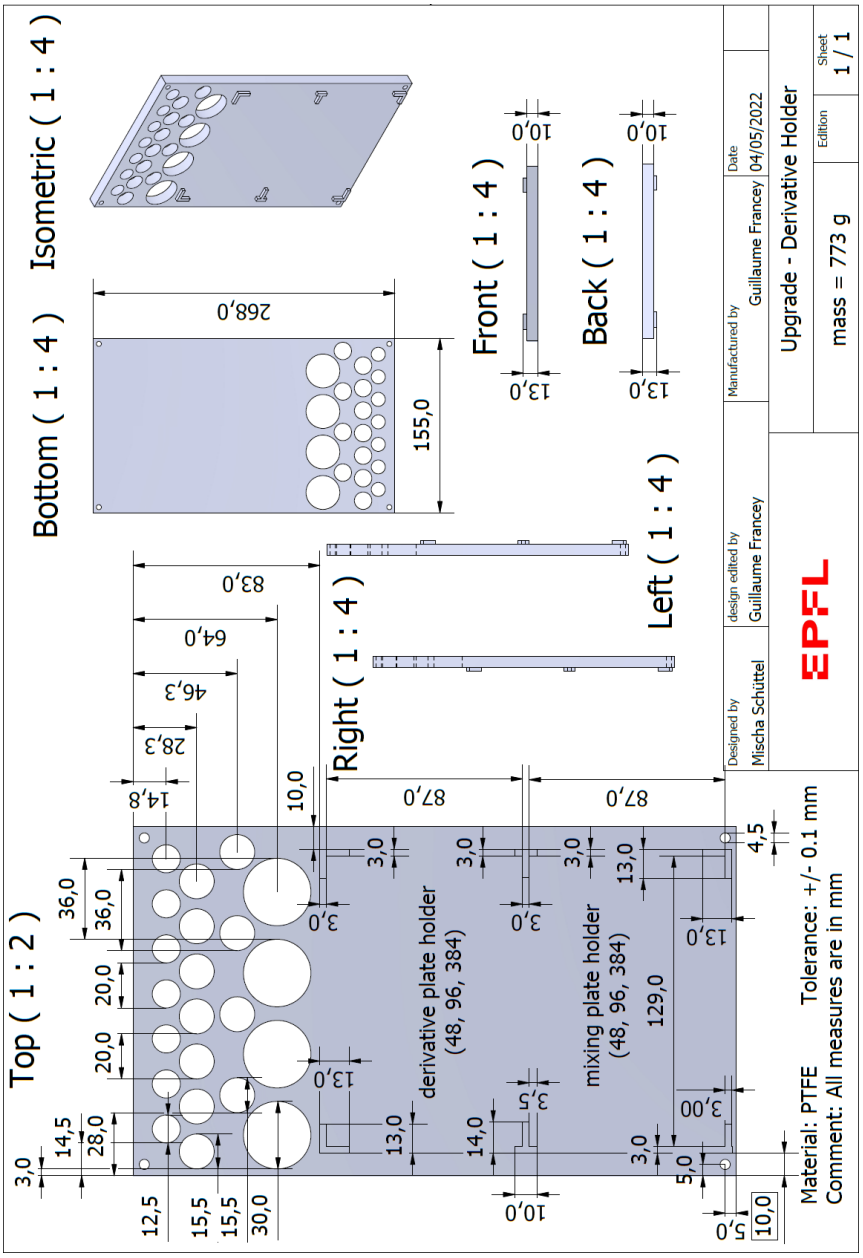
c



Supplementary Figure 2. Continued

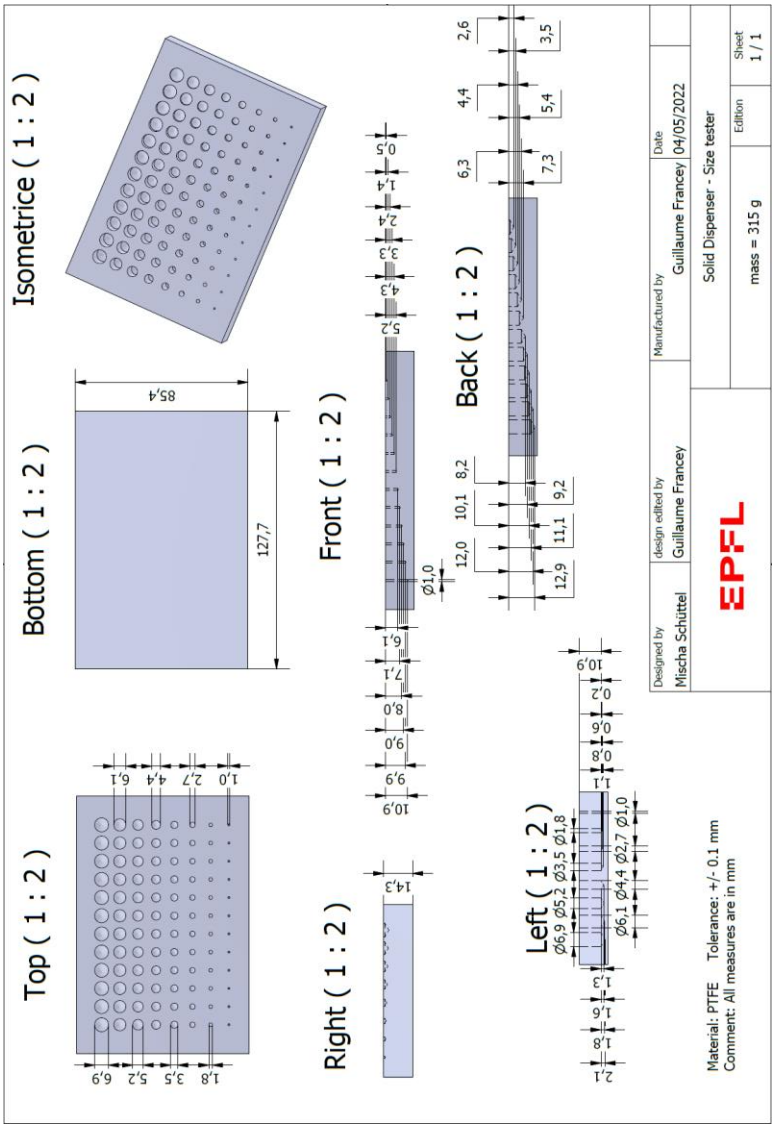


Supplementary Figure 3. Overview of the reagent rack. (a) The rack provides space for 4 × 50 ml PP canonical tubes (Greiner Bio-One, 210261), 7 × 15 ml PP tubes (Sarstedt, 60610) and two 48-well (Ritter, 43001-0062) or 96-well (Thermo Scientific, 260252) deep well plates. One of the deep well plates is used for storing the amino acids (upper) and the other for pre-activation of the amino acids (bottom). (b) The standard (left) and the modified reagent rack (right) are assembled differently using the same pieces. A = standard base plate, B and F = short spacer (2.0 cm), C = standard middle tube support plate, D = long spacer (4.0 cm), E = standard top plate for tubes, G = newly designed top plate for tubes and deep-well plates.



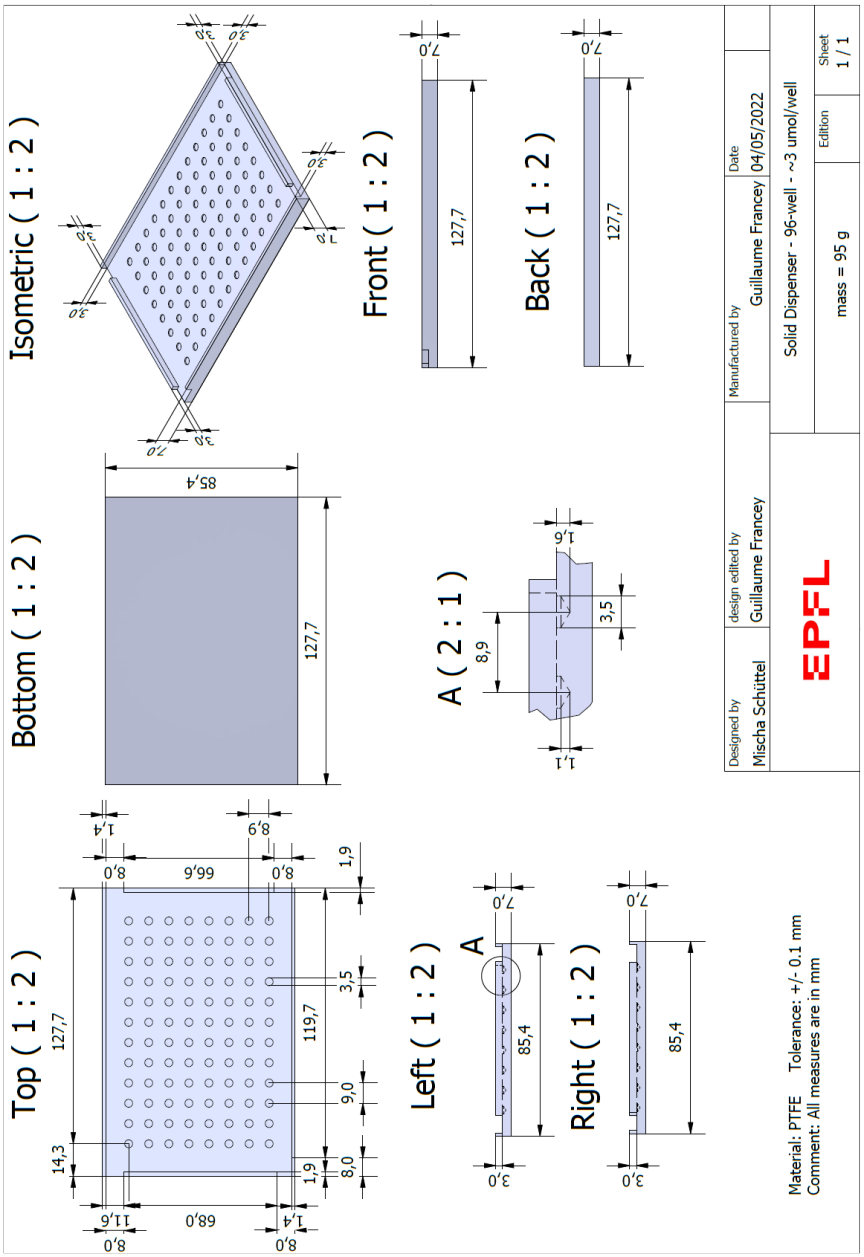
Supplementary Figure 4. Technical drawing of the new top plate for the modified reagent rack. For mounting the modified reagent rack, four longer socket head cap screws (30 vs 16 mm, M4) were used with a cylindrical head (h = 4 mm) containing an allen key fit (3 mm). See Supplementary Figure 3 for assembly.

a



Supplementary Figure 5. Technical drawing of resin dispensing tools. (a) Tester plate. (b) Dispenser for around 3 μmol resin per well for 96-well plates. (c) Dispenser for around 5 μmol resin per well for 96-well plates. (d) Dispenser for around 1 μmol resin per well for 384-well plates. (e) Dispenser for around 3 μmol resin per well for 384-well plates.

b



Supplementary Figure 5. Continued

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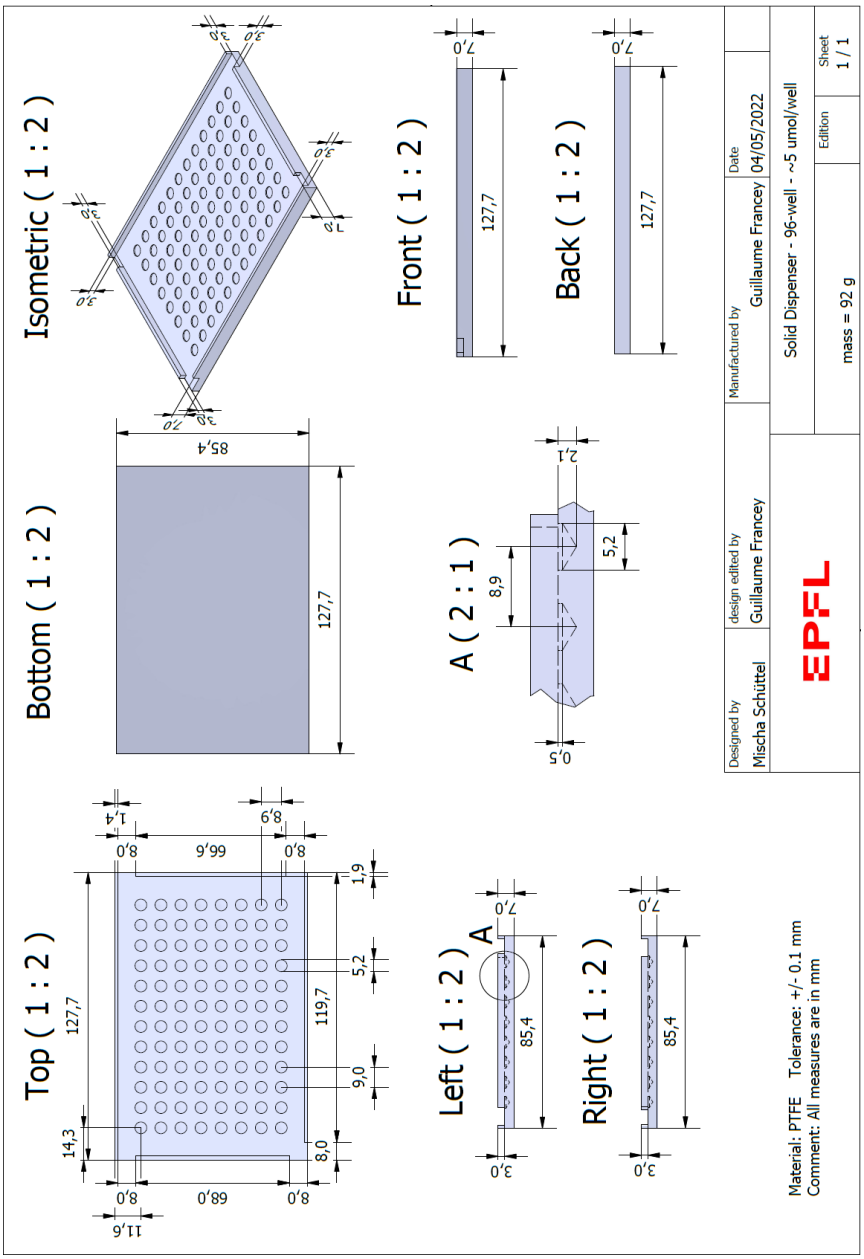
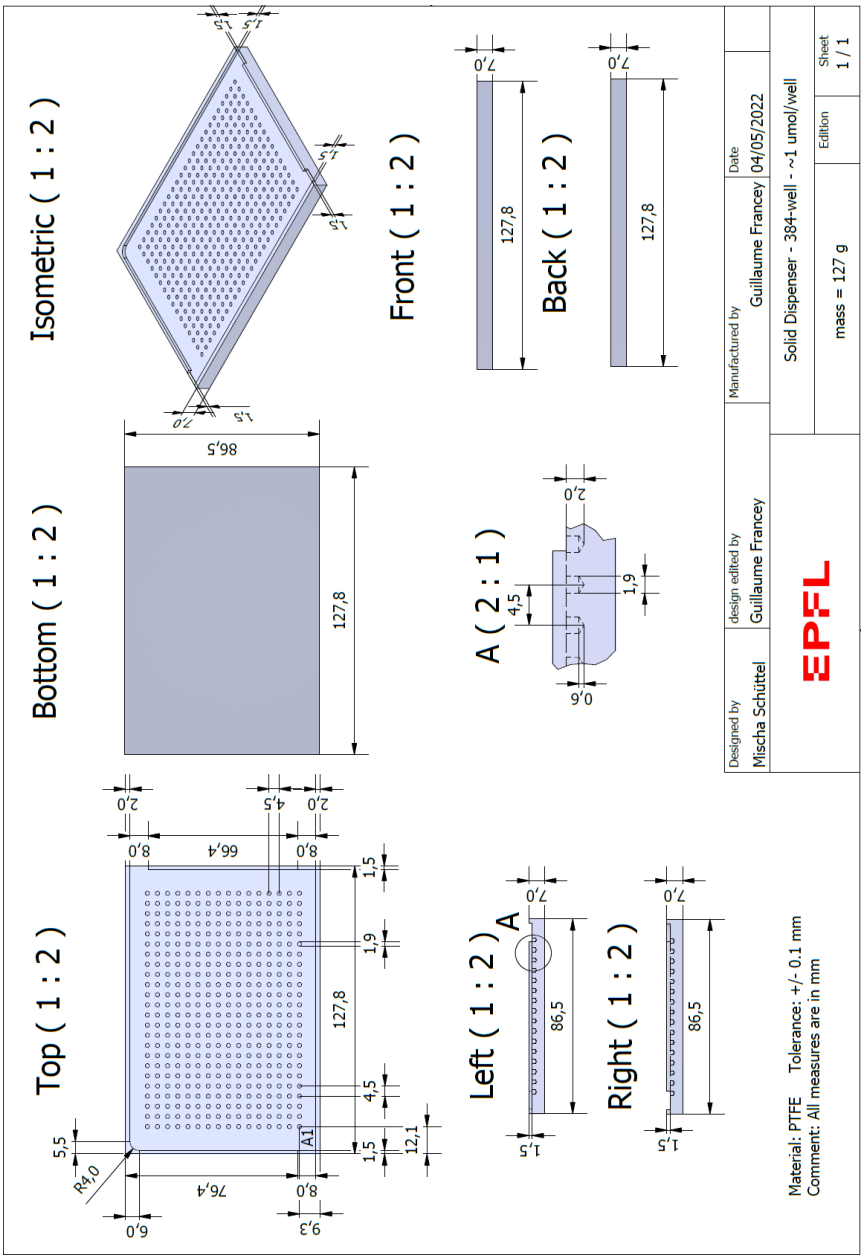


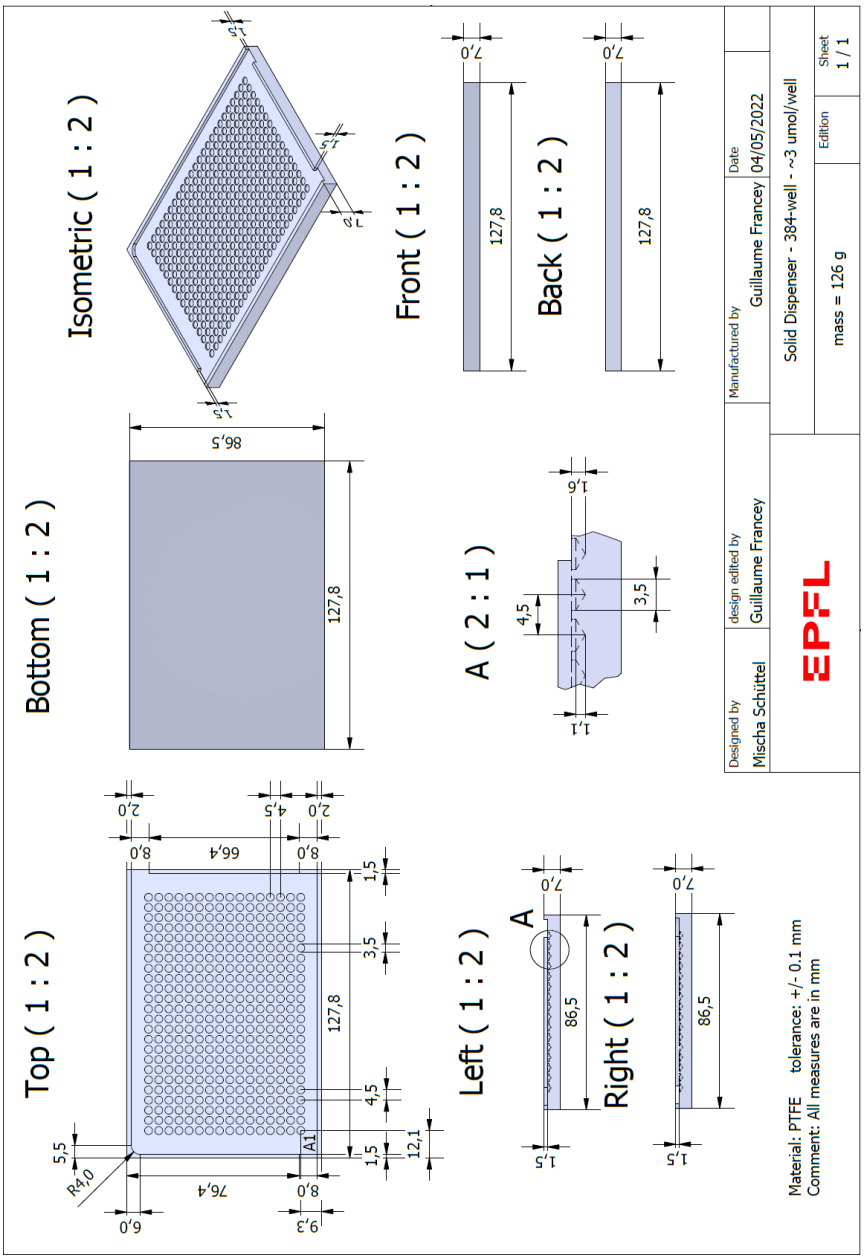
Supplementary Figure 5. Continued

d



Supplementary Figure 5. Continued

e



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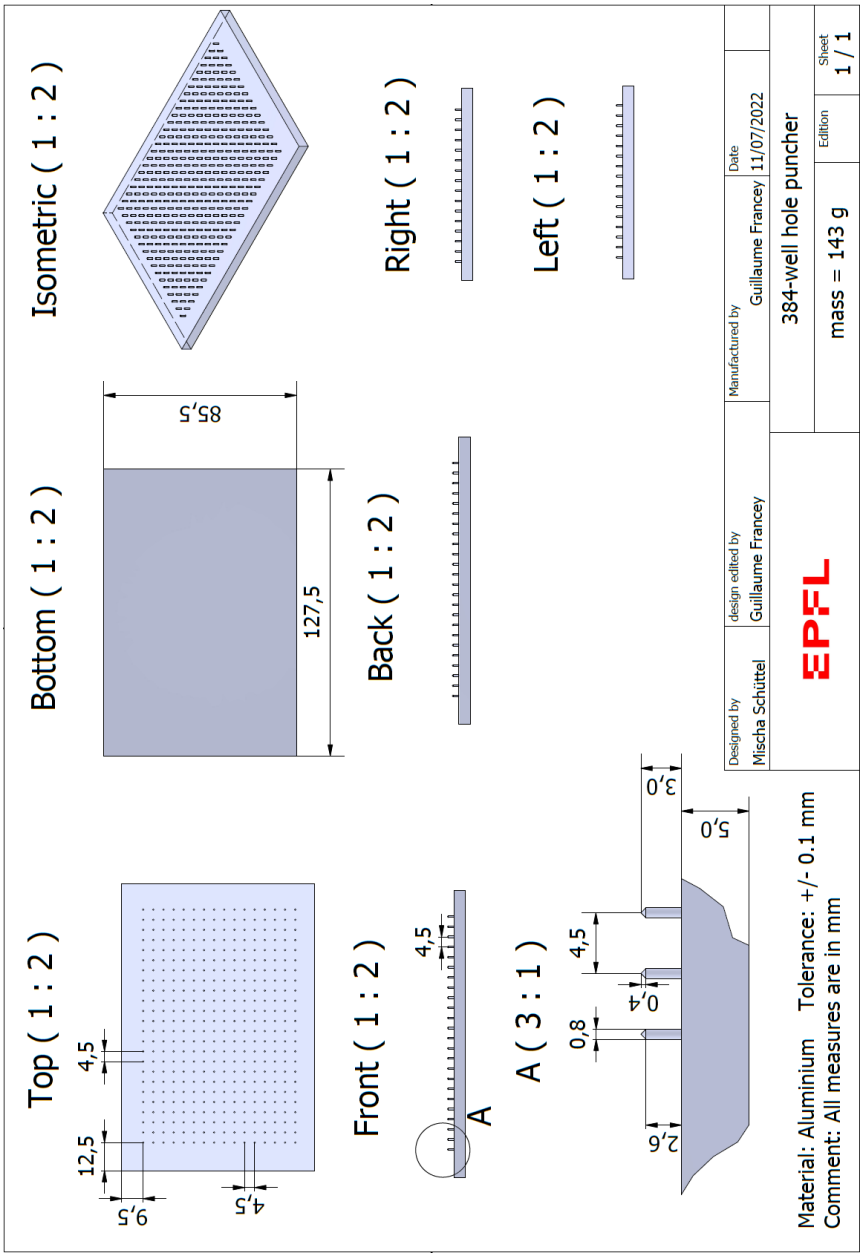
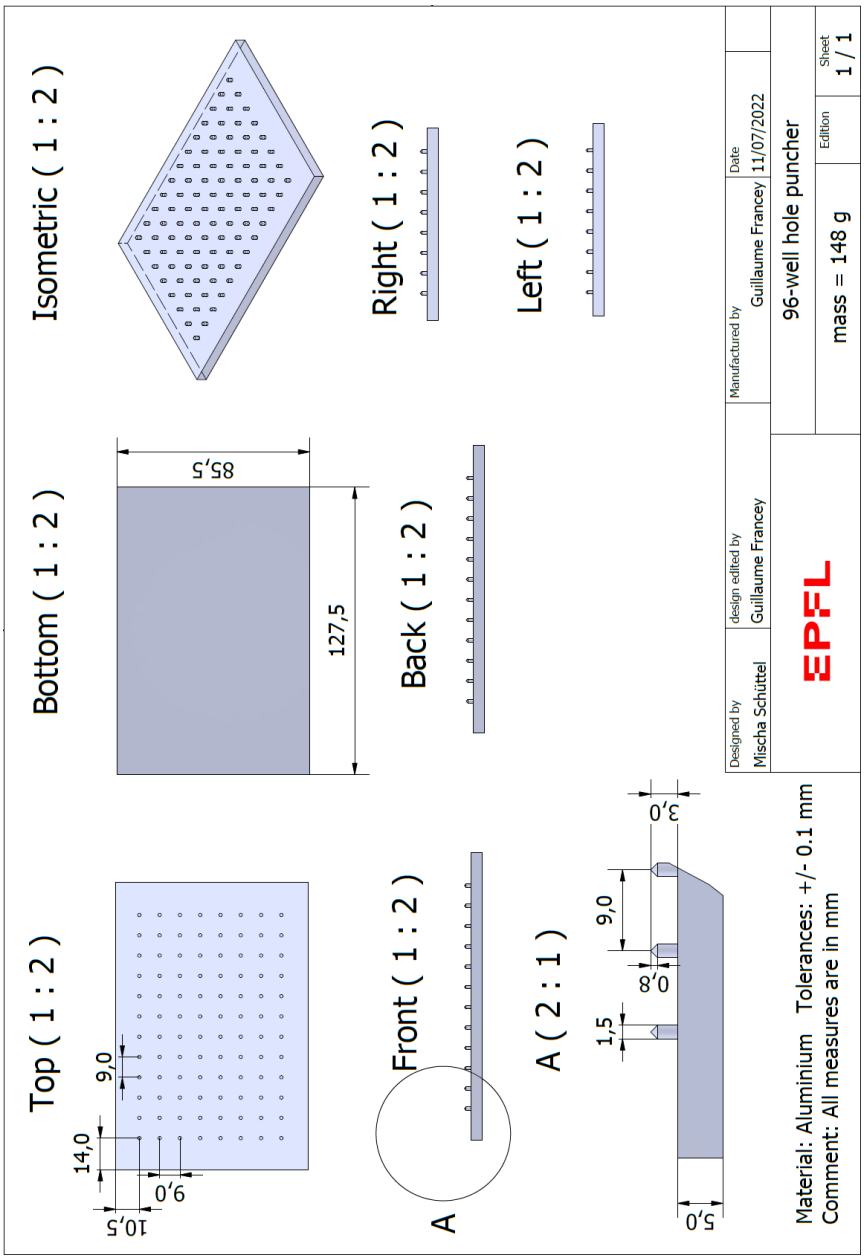
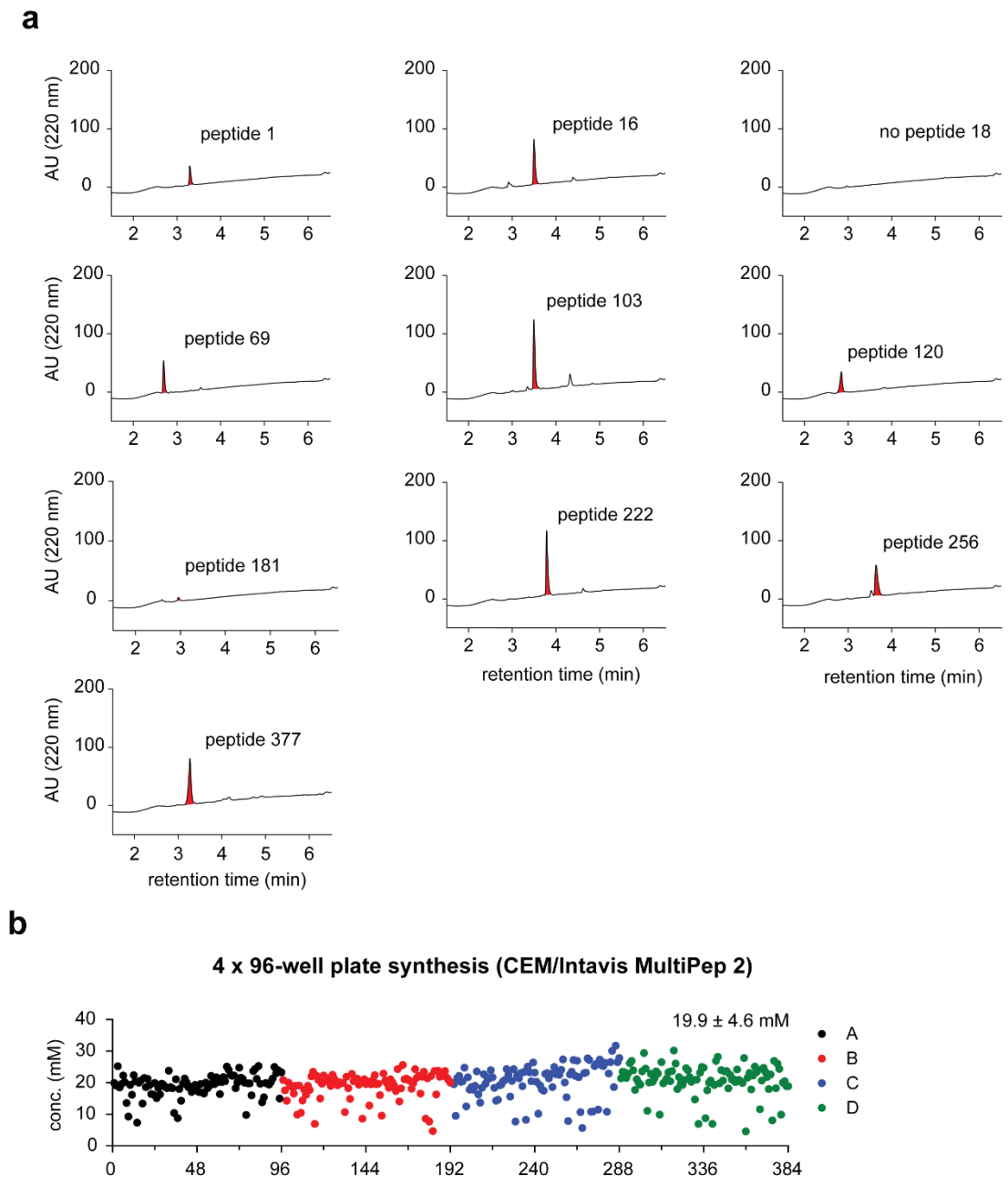


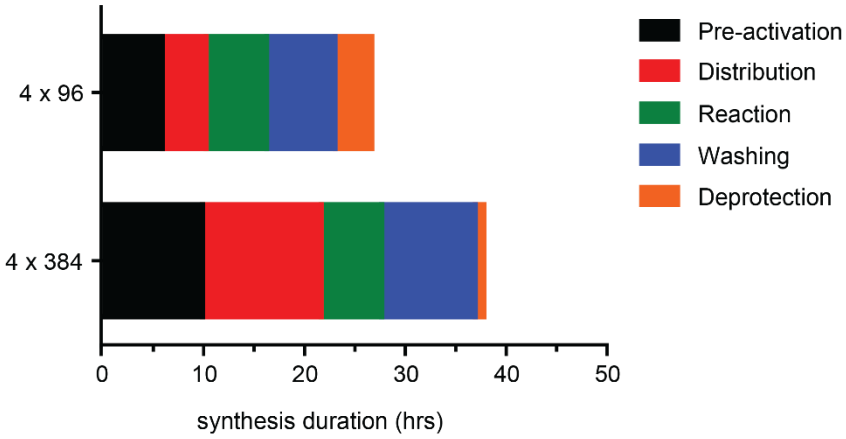
Supplementary Figure 6. Technical drawing of manual 384-well hole puncher (d = 0.75 mm, h = 3.0 mm) with sharpened tips (aluminum).



Supplementary Figure 7. Technical drawing of 96-well hole puncher (d = 0.75 mm, h = 3.0 mm) with sharpened tips (aluminum).



Supplementary Figure 8. Purity and quantity of peptides synthesized with the unmodified CEM/Intavis MultiPep 2 in 96-well plates. (a) HPLC chromatograms of 10 randomly picked peptides. (b) Concentrations of peptides were quantified by reacting the thiol groups of the peptide with Ellman's reagent and measuring absorbance.



Supplementary Figure 9. Comparison of the time required for peptide synthesis with the unmodified (4 x 96-well plates) and modified (4 x 384-well plates) setup of the CEM/Intavis MultiPep 2 peptide synthesizer. The indicated data is shown for the short random peptides described in Figure 3. Cumulative required execution time of each individual task for 4 × 96-well / 4 × 384-well plate synthesis: Pre-activation (5.9 h / 9.9 h), distribution (4.3 h / 12 h), reaction (6.0 h / 6.0 h), washing (6.8 h / 9.3 h) and deprotection (3.7 h / 0.9 h). In the synthesis in 384-well plates, the piperidine was transferred by the 16-channel manifold instead of the needle, which shortened the time substantially compared to the 96-well synthesis.