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Development of an Automatable Affinity Purification Process for DNA-Encoded Chemistry

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ABSTRACT: DNA-encoded library technologies require high-throughput, compatible, and well automatable platforms for chemistry development, building block rehearsal, and library synthesis. An affinity-based process using Watson−Crick interactions was developed that enables purification of DNA-tagged compounds from complex reaction mixtures. The purification relies on a single-stranded DNA-oligonucleotide, called *capture strand*, which was covalently coupled to an agarose matrix and to which a DNA-compound conjugate from a DNA-encoded library (DEL) reaction can be reversibly annealed to. The thus-formed DNA duplex tolerated surprisingly stringent washing conditions with multiple solvents to remove excess reactants and reagents. The tolerated solvents included aqueous buffers, aqueous EDTA solutions to remove metal ions, aqueous mixtures of organic solvents, and even pure organic solvents. The purified DNA-conjugate was eluted with aqueous ammonia and could be used for reaction analysis or for instance in DNA-encoded library synthesis. The lab equipment for purification was tailored for automation with open-source hardware and constructed by 3D printing.
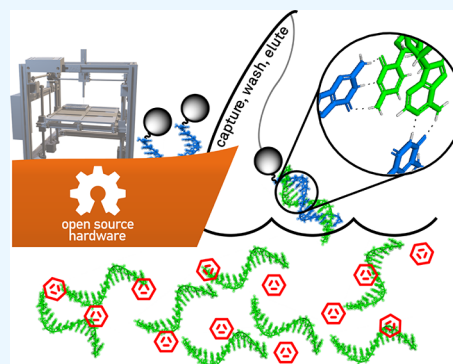
INTRODUCTION

DNA-encoded libraries (Figure 1A) are increasingly used for small molecule screening. They are synthesized by iterative enzymatic DNA-tagging and chemical compound synthesis steps that are performed in combinatorial cycles. Encoded combinatorial synthesis strategies give access to library sizes that range in 10^6−10^8-digit numbers of compounds. To this end, large numbers of DNA-tagged starting materials are mixed to complex pools. These pools of encoded starting materials are distributed to many reaction vessels. DNA-barcodes are added by ligation, and a set of building blook is coupled by a chemical reaction in parallel. Repetition of barcoding and chemistry cycles results in exponential growth of library size. (Figure 1B). Split sizes often number up to several hundred substrates that are reacted in one synthesis cycle. Translating novel reactions to an encoded library format often demands extensive experimentation campaigns to arrive at optimal reaction conditions, and the profiling of a large scope of substrates. Both reaction development and library synthesis itself would benefit from purification platforms that are compatible with high experimental throughput.[1] Currently, DNA-tagged products from reaction development and building block profiling campaigns are purified prior analysis by ethanol precipitation or by ion exchange chromatography.[2] Likewise, the product pool from a DEL synthesis cycle is mainly purified by ethanolic precipitation or more rarely by ion exchange chromatography.[3−5] Practitioners have shown ion-pair HPLC for DEL purification to improve library quality.[6] They may remove unreacted starting materials by capping with an affinity

label or using a microarray with thousands of complementary DNA sequences.[1,7] These purification methods have their strengths and weaknesses. Precipitation is a parallelizable process but may lead to a loss of material due to partial oligonucleotide solubility in ethanol/water mixtures.[8,9] Conversely, buffer components, such as salts, but also hydrophilic building blocks from a reaction may precipitate with the DNA, leading to DNA contamination and reagent carry-over to the next synthesis cycle or may render reaction analysis more complicated.[10,11] Furthermore, precipitation is not easily automatable. Ion exchange chromatography allows for stringent removal of excess reagents by simple washing steps but requires high salt concentrations for product elution that need to be removed from the DNA.[12−14] HPLC purification requires sufficient retention time differences for the product pools versus the starting materials; it is therefore an option for the first synthesis step in a DEL synthesis or may be limited to combination with Fmoc-chemistry.[6,10,15] Finally, to purify the DNA-encoded library via affinity labeling or other bead-based purification methods, such as carboxylate beads, the DEL must contain these chemical components for purification, e.g., free
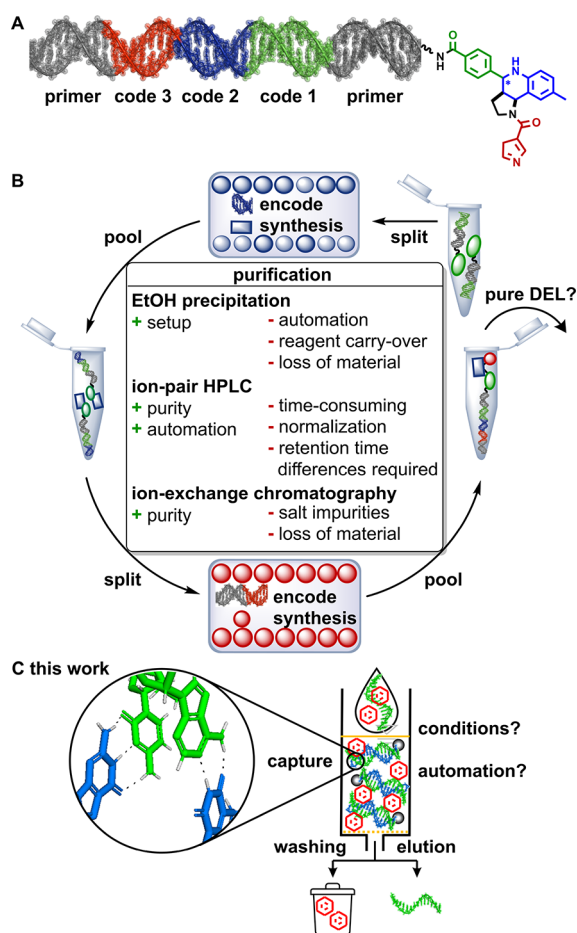
Figure 1. Purification techniques in DNA-encoded library technology. (A) Representative DNA-encoded compound. (B) DNA-encoded libraries are synthesized by split-and-pool combinatorial chemistry with alternating DNA-tagging, chemical synthesis, and purification steps. Conventional purification techniques are ethanol precipitation, ion exchange chromatography, and ion-pair chromatography. (C) This work shows a new purification method based on Watson−Crick interaction of two single-stranded DNA strands combined with solid-phase extraction that uses the principle of capture, washing, and elution.

primary amines. This is very dedicated and not feasible for all DEL designs.[1,16]

Here, we present an affinity/Watson−Crick interaction-based platform for purification of single-stranded DNA-conjugates (Figure 1C). It was inspired by the well-established process for mRNA purification with oligo-dT beads.[17−19] We were surprised that it allowed for stringent removal of contaminants with selectable organic solvents, and elution of the DNA-conjugates was effected by a short incubation with either dilute aqueous NaOH solution or ammonia solution. Advances in 3D printing and a decrease in the cost of the printer equipment as well as the polymer material required for printing have made this technology accessible to many laboratories and led to large improvements in the design of do-it-yourself (DIY) labware that can be adapted to specific needs.[20−23] Programming and control of the self-made lab equipment for chemical and biochemical experiments are performed by open-source hardware and software.[24−28] We combined the newly established purification process with tailored 3D-printed lab equipment that allowed for paralleliza-

tion and optional automation of DNA-conjugate purification, based on Watson−Crick interaction of two single-stranded DNA strands combined with solid-phase extraction that uses the principle of capture, washing, and elution.

■ RESULTS AND DISCUSSION

Development of the Affinity-Based Purification Protocol. The affinity-based purification process presented here is based on the principle of a disperse solid-phase extraction (DSPE), which is a special form of the solid-phase extraction (SPE). In DSPE, the solid phase is not spatially fixed, for example, in a column or in an array, but it is dispersed in a solution (Figure 2A). We used here a dispersible agarose matrix with a covalently bound azide functionality. A 14mer-DNA single strand **DNA-1** (5′-GAA TTC GGC AAG AC-3′) was loaded with low density (**DNA-1**: 1 nmol versus N_3-functionalities on the agarose matrix: 150 nmol) to the matrix by copper-catalyzed alkyne−azide cycloaddition reaction (CuAAC) to obtain **capture-DNA-1** (Figure 2B). A second DNA single strand with a fluorophore label **DNA-2** was used as in-process control. After oligonucleotide immobilization, the matrix was washed on a filter with water and aqueous EDTA solution. The in-process control showed a yellow staining of the matrix (**capture-DNA-2**), indicating successful DNA immobilization (Figure 2Bb and Figure S2). In the negative control experiment without the addition of copper and ligand, the matrix was colorless as expected; thus, non-specific binding of **DNA-2** to the matrix can be ruled out (Figure 2Bc and Figure S2).

In DSPE, the solution containing the products to be purified, here the complementary DEL-DNA-single strand **DNA-3** (5′-GTC TTG CCG AAT TC-3′), is added to the dispersion with the sorbent and mixed (Figure 2Ca−c). In a first experiment, 500 pmol of the **DNA-3** was added to the **capture-DNA-1** (0.5 mg matrix; **DNA-3**: 500 pmol versus **DNA-1** on matrix: 1 nmol). **DNA-3** was bound to the solid phase by a form of adsorption, in this case by annealing via Watson−Crick interactions. Certain conditions are conducive to annealing, which can be modulated by tempering and buffer composition. Temperatures of 25−95 °C were tested without observing significant differences in annealing efficiency (Figures S5 and S19) and different buffer solutions can be used, e.g., annealing buffer, T4 ligase buffer, nuclease-free duplex buffer (details on the buffer compositions are given in the SI). The contaminants, for example, excess reagents, reactants, catalysts, and additives, can then be separated from the product by simple filtration. Initially, we intended to wash the matrix with aqueous buffer solutions that are known to preserve the Watson−Crick interaction and then to elute the purified DNA product with aqueous mixtures of organic solvents. Surprisingly, we were not able to elute the fluorescence-labeled **DNA-3** from the matrix by washing with organic co-solvents (Figure 2Cd and Figure S4). This is in conflict with observations that dissolving duplex DNA in aqueous mixtures of polar organic solvents or in pure organic solvents (formamide, methanol, DMSO, pyridine) led to duplex destabilization, unfolding, and strand separation.[29−31] Actually, we planned to use the more readily available ssDNA as capture-DNA only in initial experiments to establish the purification method and then to replace the capture-DNA by a peptide nucleic acid (PNA) in order to exploit the increased duplex stability of hybrid DNA−PNA duplexes and thus a wider range of washing conditions.[29] Since our experimental
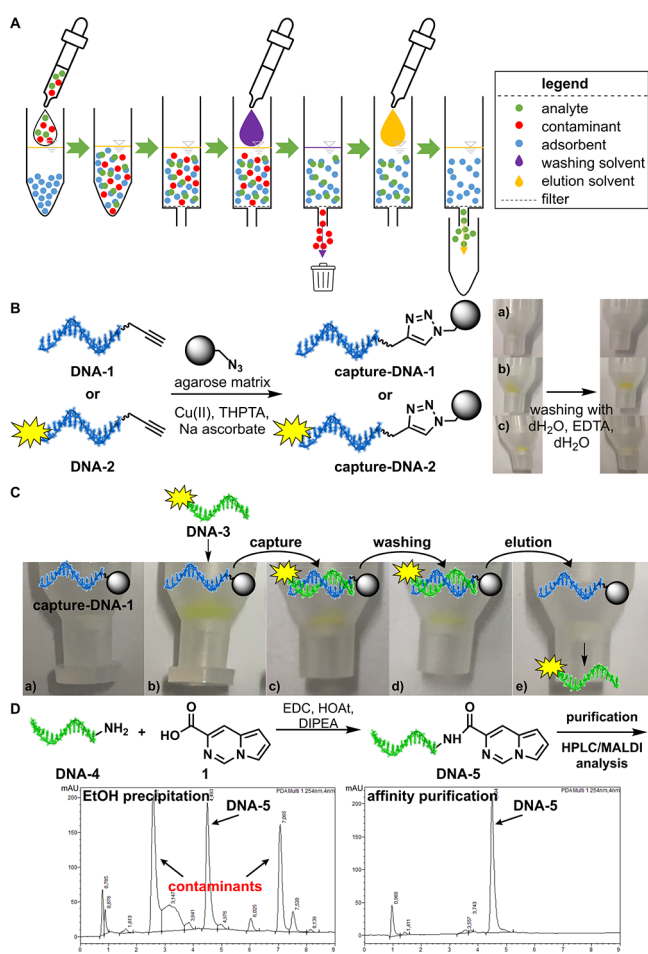
Figure 2. Affinity-based purification process for DNA-encoded chemistry. (A) Schematic presentation of the disperse solid phase extraction process. (B) Preparation of the **capture-DNA-1** and control **capture-DNA-2** via the CuAAC-chemistry. Pictures of the **capture-DNA-1** (a) and **2** (b) in filter columns after the CuAAC-reaction (left-hand photo) and after washing (right-hand photo) with water and aqueous EDTA (0.1 M) to remove residues from the CuAAC-reaction. **DNA-2** was labeled with a fluorophore as in-process control. A control (c) was performed without the reagents Cu/THPTA/Na-ascorbate with **DNA-2** to exclude unselective binding of the DNA to the matrix. (C) Pictures of the **capture-DNA-1** in filter columns (a) after capturing **DNA-3** (b,c), washing with several solvents (d), and elution of **DNA-3** (e). **DNA-3** was labeled with a fluorophore as in-process control. (D) Comparison of the purification of amide coupling product **DNA-5** by ethanolic precipitation (left-hand HPLC chromatogram) and affinity purification (right-hand HPLC chromatogram).

results clearly showed that the agarose matrix with the duplex DNA composed of **DNA-1** and **DNA-3** could be washed with water, aqueous EDTA-solution to remove metal contaminants, and various aqueous mixtures of organic solvents (EtOH, MeOH, MeCN) as well as pure organic solvents (DMF, MeOH, MeCN, CH_2Cl_2) to remove contaminants of different polarities and solubilities by vacuum or centrifugation, we kept the DNA as a capturing unit. The potential loss of the product **DNA-3** was monitored using the fluorescence label. After individual washing steps, the yellow staining of the matrix attributable to **DNA-3** was always observed (Figure 2Cd). Then, the product **DNA-3** was eluted from the affinity matrix via denaturing of the double strand and DNA recovery was

determined by measuring the UV−Vis absorption with NanoDrop and by analytical HPLC. Heat denaturation was attempted first for DNA elution. For this purpose, the matrix was suspended in water without salt and heated to up to 95 °C. By rapid cooling (ice bath) and direct collection of the supernatant, the purified **DNA-3** should have been eluted. To our surprise, no **DNA-3** could be recovered after heat denaturation. This could also be observed from the persistent yellow staining of the matrix. Next, we tested different aqueous mixtures of organic solvents (EtOH, MeOH, MeCN) in combination with heat denaturation. However, these experiments did not lead to DNA elution too (Figures S7−S9). Only addition of aqueous NaOH solution (0.1 M) to the matrix led to quantitative DNA-elution visible through the colorless matrix (Figure 2Ce and Figures S10, S12, and S13), UV−Vis absorption measured by NanoDrop, and HPLC analysis of the eluent. Since alkaline conditions (pH 12) caused disruption of the Watson−Crick interaction in this system, we tested elution with aqueous ammonia solution (32%) as this eluent could be removed after elution by simple evaporation. Indeed, the concentrated ammonia solution effected elution of **DNA-3** as detected by decolorization of the matrix, measurement of the UV−Vis absorption, and HPLC analysis of the eluent (Figure 2 and Figures S14 and S15). To exclude non-selective binding of the complementary **DNA-3** single strand to the agarose matrix, it was added to an agarose matrix, which was not loaded with the **DNA-1**. As expected, after mild washing only with aqueous buffer, the staining of the matrix was completely lost, and we could also not detect any **DNA-3** in the subsequently applied alkaline elution buffer by UV−Vis spectrometry and HPLC analysis (Figures S17 and S18). After the successful setup of the purification method, it was optimized for the maximal recovery of the purified DNA. For this purpose, the amount of **capture-DNA-1** matrix (0.5 to 1.5 mg; 1 mg matrix equals 2 nmol of capture-DNA) and the amount of **DNA-3** were varied from sub- to superstoichiometric amounts versus the **DNA-1** on the matrix (**DNA-3**: 500−3000 pmol versus **DNA-1**: 1 nmol). It was found that the recovery increased with increasing amount of **capture-DNA-1** and decreased again with increasing amount of **DNA-3**. Optimal conditions were found to be 500 pmol of **DNA-3** and 1.5 mg of **capture-DNA-1** corresponding to 3 nmol of the capture-DNA (Figure S21). Finally, we compared the affinity matrix purification to ethanolic precipitation-based purification of a typical DEL-reaction. To a single strand **DNA-4** with an C6-aminolinker the carboxylic acid **1** was coupled with HOAt, EDC·HCl, and DIPEA (Figure 2D).[32] Afterward, the product **DNA-5** was purified by ethanolic precipitation or by affinity purification. The purified **DNA-5** was analyzed using MALDI-MS and analytical HPLC. The ethanolic precipitation gave the product peak in the HPLC chromatogram at 4.5 min but also two prominent peaks at 2.5 and 7.1 min, which can be attributed to low-molecular-weight impurities, e.g., the carboxylic acid **1** precipitated from the ethanolic solution and eluted at 2.5 min (Figure 2D and Figure S22b). Furthermore, clear salt adducts can be observed in the MALDI-MS spectrum (Figure S22c). In comparison, a clean HPLC chromatogram (Figure 2D) and also MALDI-MS spectrum (Figure S22d) of the desired **DNA-5** could be obtained by affinity purification.

Microwell Filter Plate Device for Automated Purification.

The repetitive and laborious washing and elution steps of the newly established DNA-conjugate purification process
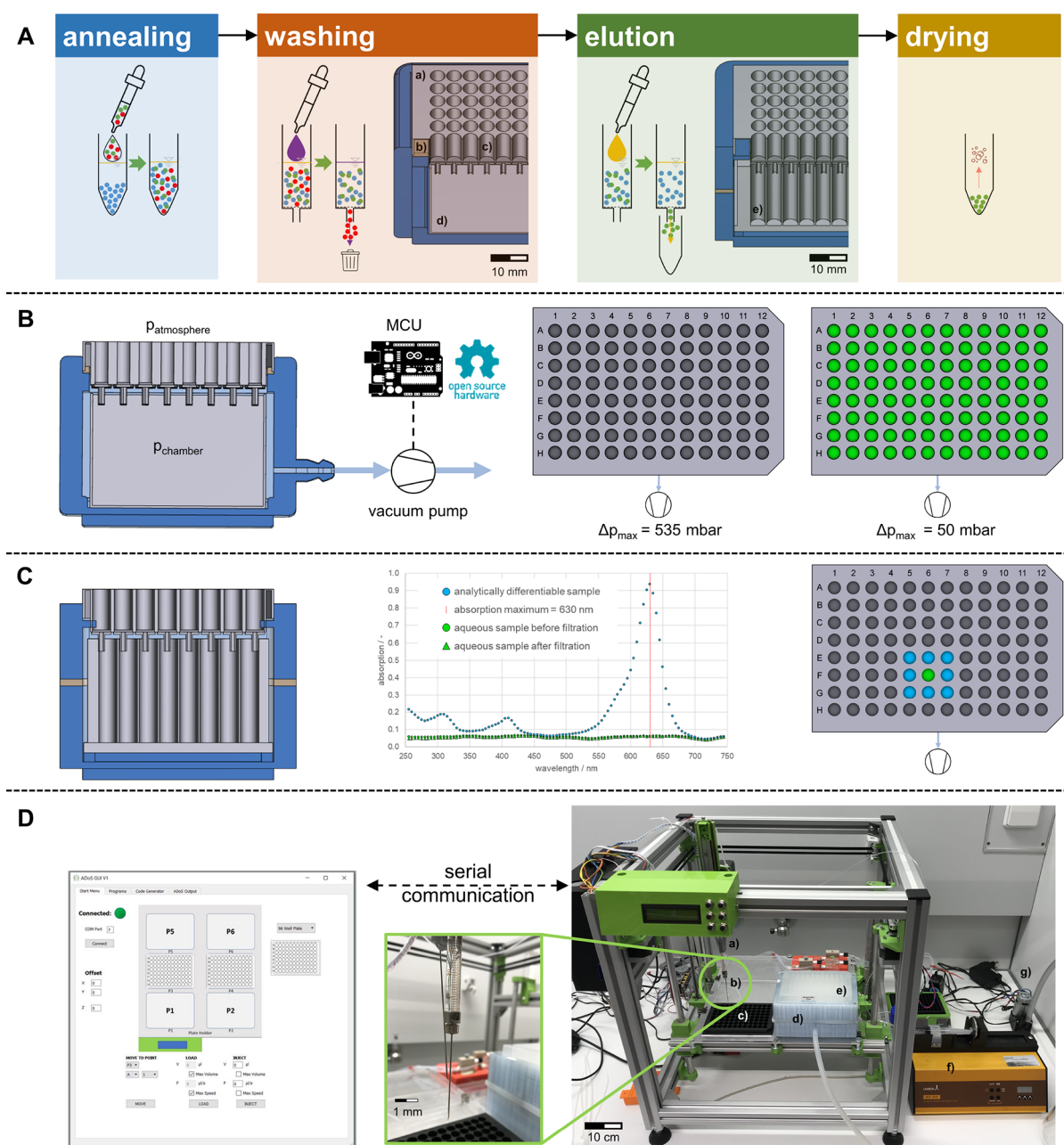
Figure 3. Design, testing, and automation of the microwell filter plate device. (A) Design of the tailored 3D-printed vacuum chambers for washing and elution with (a) a 96-microwell filter plate with 35 μm pore size; (b) silicon sealing; (c) filter; (d) waste container; (e) 96-microwell plate for collecting the cleaned product **DNA-3**. (B) Testing of the required pressure difference and leak-tightness between $p_{chamber}$ and $p_{atmosphere}$ using an open-source vacuum pump with an MCU. Loaded filter positions with 500 μL of aqueous sample are marked in green and empty positions sealed with foil are marked in light gray. (C) Excluding cross-contamination of probe (green) and adjacent microwell filter plate positions (blue). (D) Overview of the experimental setup of the automated dosage system (ADoS) with: (a) automated injection unit (AIU) with a GC syringe; (b) close up of injection units with needles for dH$_2$O and organic washing solvents; (c) microwell filter plate with solvents used; (d) vacuum chamber for washing; (e) microwell filter plate; (f) syringe pump with syringe for dH$_2$O; (g) open-source vacuum pump.

(Figure S23 and Figure 2A) offer great potential for parallelization and automation with tailored 3D-printed lab equipment in combination with robotics. Therefore, a microwell filter plate device (Figure 3A) was developed consisting of a 96-microwell filter plate with 35 μm pore size, a 3D-printed vacuum chamber, and an open-source vacuum pump with a microcontroller unit (MCU). The tailored lab equipment was designed by computer-aided design (CAD) software to produce technical drawings for fused deposition modeling (FDM) 3D printing with polylactide (PLA) on an

Ultimaker S5 3D-printer. The technical drawings and experimental setup can be found in the SI (Figures S24 and S25).

The washing and elution steps in the DNA-conjugate purification process required two differently constructed vacuum chambers. The first vacuum chamber for washing was a single-part construction, as the filtrate was discarded into a waste container. The product **DNA-3** connected to the beads by Watson−Crick interaction remained on the 96-microwell filter plate during washing steps. For elution, the filter plate was
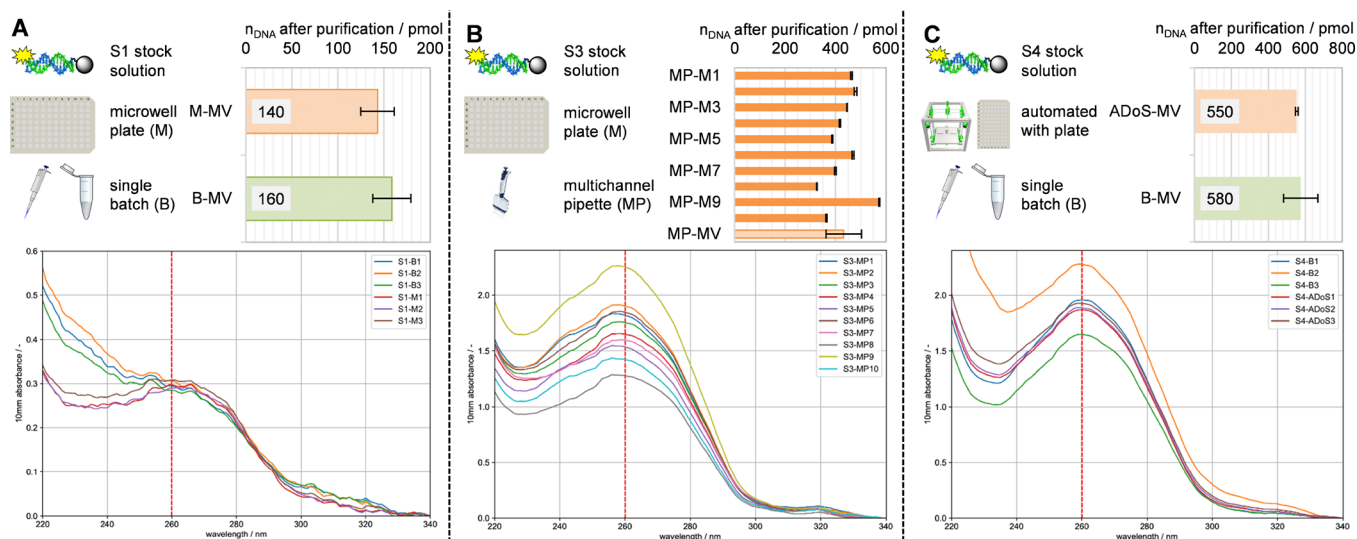
Figure 4. Evaluation of the automatability of the DNA-conjugate purification process in three individual experiments. (A) Comparison of the microwell filter plate device (M) and single batch procedure (B) with stock solution S1. (B) Validation of the parallelization of the microwell filter plate device with the tailored 3D-printed equipment using stock solution S3 and a multichannel pipette (MP) manually. (C) Validation of the automated microwell filter plate washing steps using stock solution S4.

moved to a second vacuum chamber. This vacuum chamber was a two-part construction for collecting the filtrate with the purified DNA-conjugates into a 96-microwell plate. After construction, we evaluated and examined the 3D-printed vacuum chambers by the following aspects to confirm the error-free functionality of the microwell filter plate device for DNA-conjugate purification. Especially, it was important to ensure that the design of the vacuum chambers prevented cross-contamination.

First, the leak-tightness of the vacuum chambers and generation of the required vacuum inside the chambers were investigated by a differential pressure test (Figure S26). This test indicated that the open-source vacuum pump with an MCU was able to generate a maximal differential pressure of 535 mbar for an unloaded sealed 96-microwell filter plate. Additionally, it was checked how different filter positions on the 96-microwell filter plate loaded with 500 μL of aqueous sample had an influence on the vacuum. Additionally, it had to be guaranteed that the washing solvents were completely drawn through the filter within a known operating time of the vacuum pump.

The results of the differential pressure test for a loaded 96-microwell filter plate showed that the differential pressure varied depending on the number and position of the aqueous sample, as shown in Figure 3B. For a single loaded filter position, the measured differential pressure ranges from 163−187 mbar, and the aqueous sample was completely sucked through the filter after 3 s, regardless of the position on the 96-microwell filter plate. Filtration of multiple loaded filter positions in a row or column on the filter plate required a slightly longer time of 4−5 s, and the measured differential pressure was in the range of 67−74 mbar. For a fully loaded 96-microwell filter plate with 96 aqueous samples, the vacuum pump built up a differential pressure of 50 mbar. However, a significantly longer time for filtering all samples was not observed. An investigation in the pressure difference for each position on the 96-microwell filter plate is given in Figure S26. As an interim conclusion, the results in Figure 3B confirmed that the tailored 3D-printed vacuum chambers fully achieved

the leak-tightness and required vacuum, and the influence of the sample position and the number of samples on the 96-microwell filter plate was negligible for this purification process.

Second, it was crucial to prevent the occurrence of cross-contaminations, when purifying multiple different DNA-conjugates simultaneously on the same 96-microwell filter plate. For evaluation of the cross-contamination, a simple experiment using a dye was performed in Figure 3C. A colorless aqueous sample to be analyzed was filled into position F6 of the 96-microwell filter plate, and all adjacent positions were filled with analytically differentiable solution of blue aqueous samples, varying significantly in the absorption maximum with a peak at the wavelength of 630 nm. A quantitative UV−Vis measurement ensured that the aqueous sample after filtration did not indicate the characteristic peak of the analytically differential sample. The occurrence of cross-contamination by adjacent positions was excluded, since no measurable peak at the wavelength of 630 nm appeared in the aqueous sample after filtration. Thus, we successfully verified the error-free functionality of the tailored 3D-printed purification equipment by evaluating leak-tightness and excluding cross-contamination.

Next, we evaluated the automation capability of the laborious and repetitive washing steps of the newly established purification process with the 3D-printed equipment. For the automated purification, a previously developed automated dosage system (ADoS) by Bobers et al.[33] was used as a completely open-source and off-the-shelf system based on 3D-printed equipment. The ADoS workspace consisted of six flexible positions with the dimensions of a standardized 96-microwell plate (127.76 mm × 85.48 mm).[33] This design used self-made and 3D-printed modules in the same dimensions as the developed vacuum chamber in this work. The modified workspace of the ADoS for washing with a 96-microwell filter plate is shown in Figure 3D. It consists of a 96-microwell plate with the five washing solvents (aqueous EDTA, DMF, MeOH, MeCN, CH_2Cl_2), the designed vacuum chamber for washing, and a cleaning station for continuous cleaning of the

automated injection unit (AIU). The AIU in this work consisted of a GC syringe controlled by a stepper motor for dosing of dissolved chemicals and the newly added fixed needle for dosing dH$_2$O by an external syringe pump. This step was necessary because we performed iterative washing steps with organic solvents and water. An exemplary sequential function chart of the automated washing steps and all technical details are given in Figure S27.

The performance of the microwell filter plate device with the tailored 3D-printed equipment was compared to the conventional single batch procedure by purification of the test samples **DNA-3**. The microwell filter plate device was validated for parallelization and automation with the ADoS. Experimental results were obtained using the newly developed DNA-conjugate purification process with fresh stock (S1, S3, S4) solution for each experiment. Qualitative results regarding the purity of the **DNA-3** product after purification were obtained by measuring the unitless absorbance spectrum from 230 to 280 nm with the absorption maximum of DNA at 260 nm. After purification, the quantitative recovery parameter for the following experiments was the amount of **DNA-3** substance in pmol calculated from the A260 absorbance. The mean value with the corresponding standard deviation was determined by performing the purification three times per sample.

In a first set of experiments, we compared the recovery of **DNA-3** purified by manual pipetting using a single well on the microwell filter plate and microliter filter columns, with which the whole process was set up (Figure 4A). The head-to-head comparison of the quantitative results of these experiments showed that both the single batch procedure and the microwell filter plate device provided similar recovery of the **DNA-3**. The mean values of the amount of purified **DNA-3** were 140 ± 20 pmol for the microwell filter plate and 160 ± 20 pmol for the batch filter column reference. The mean value for the microwell filter plate was slightly lower than for the batch filter column, but the amounts of purified **DNA-3** with the tailored equipment were within the precision range of the reference single batch filtrations. Together, these results show the applicability of the self-built equipment with a microwell filter plate within a precision range of more than 15%, which was fully satisfactory for the purification process.

Next, we validated the microwell filter plate device for parallel purification of multiple **DNA-3** products, and we investigated whether parallel pipetting has an impact on purification precision. A multichannel pipette parallelized washing and elution steps on the 96-microwell filter plate loaded with **DNA-3** products out of stock solution S3. As shown in the quantitative absorption spectra (Figure 4B), all 10 **DNA-3** products were successfully purified, and no contaminations of the **DNA-3** with absorption maxima at 280 or 230 nm were visible in the UV−Vis spectrum. This finding validated the functionality of the tailored 3D-printed purification equipment for multiple **DNA-3** products. The calculated mean value of the amount of recovered and purified **DNA-3** was 430 ±70 pmol, and the precision was only slightly lower than the precision that was achieved with a single-channel pipette in the previous experiments. This slightly lower precision in the parallel process can be explained by two outlier samples M9 and M10 (Figure 4B). These experiments showed the applicability of the tailored equipment for purification with multichannel pipettes that offer enormous potential for time-consuming pipetting steps and advanced automation.

Finally, in a proof-of-concept experiment, we demonstrated the potential of our 3D-printed microwell filter plate device for automation using the ADoS. To this end, the washing vacuum chamber was successfully integrated into the ADoS workspace. The results for automated washing of the **DNA-3** products with the washing solvents are shown in Figure 4C. The head-to-head comparison of the quantitative results of these experiments showed that the ADoS reproduces the manually performed washing steps with the same amount of purified **DNA-3** products. The amount of purified **DNA-3** was in a valid precision range for manually batch purification (580 ± 90 pmol). The quantitative absorption spectra confirmed the successful automated purification of DNA-conjugate because no contaminations absorbing at 280 or 230 nm were visible. However, the automated washing in the microwell filter plate was three times longer than manual single batch washing due to the design of the ADoS. In order to avoid plastic waste in comparison to manual pipetting with disposable tips, the AIU uses a fixed installed needle in combination with a cleaning station, which requires additional time between pipetting steps. This extra effort for cleaning the syringe was essential for direct comparison of the results with manual pipetting. The advantages of automated purification are given by an overnight operation and the integration with other steps of DEL synthesis, despite a longer operating time. In conclusion, the 96-microwell plate filter device in combination with automated pipetting was applicable for the purification of DNA-conjugates in the microliter range.

■ CONCLUSIONS AND OUTLOOK

Purification systems that allow for stringent removal of impurities from DNA-tagged compounds are of high value for DNA-encoded library technology. In this contribution, we showed an affinity-based purification process with application potential to DNA-encoded chemistry. The purification relies on immobilization of a short 14mer-DNA oligonucleotide that is fully complementary to the partial sequence in a given DEL barcode construct. Much to our surprise, the Watson−Crick interactions on the agarose matrix tolerated highly stringent washing conditions with multiple solvents. These included aqueous buffers, aqueous EDTA solutions to remove metal ions, aqueous mixtures of organic solvents (that were initially intended for DNA elution), and even pure organic solvents. The stability of a DNA duplex in organic solvents has to date been described for oligonucleotides that were immobilized on ion exchange resin.[4] In our system, the DNA products could only be eluted under alkaline conditions, thus providing highly pure DNA products. The use of aqueous ammonia solution offers the option to control the solvent in DEL synthesis processes or for DEL storage as this eluent can be removed by simple evaporation. The process was robust enough to be parallelized in a tailored 3D-printed equipment and the laborious washing steps could even be automated. We see potential applications of this process in the development of chemical methods on ssDNA substrates for DEL design and for the purification of DELs that are encoded with ssDNA oligonucleotides.[5,34−36] The system could also be used for the purification of DELs that are encoded with dsDNA by simply adding a single-stranded region to the DEL barcode, e.g., in or adjacent to a primer region. Clark et al. used a closing primer, which had a single-stranded region. This single-stranded region could be used for the affinity purification of the DEL as a final step.[10]

EXPERIMENTAL SECTION

Materials. Azide agarose was purchased from Sigma-Aldrich (Taufkirchen, Germany). DNA in solution was purchased from Integrated DNA Technologies (Leuven, Belgium). 5′-Aminolinker-modified DNA oligonucleotides attached to controlled pore glass solid phase (CPG, 1000 Å) were synthesized by Ella Biotech GmbH (Fürstenfeldbruck, Germany). The agarose matrix was washed in Mobicol "Classic" from Mo Bi Tec GmbH (Göttingen, Germany) with a filter pore size of 35 μm or in filter columns (X-Col, HTI Automation, Ebersberg, Germany) with a filter pore size of 7−12 μm.

Instruments. The controlled pore glass solid phase was filtered on a synthesis column plugged onto a VM20 Vacuum Manifold (Sigma-Aldrich). Oligonucleotide-small molecule conjugates were purified by ion pair reverse-phase high-pressure liquid chromatography (HPLC, Prominence Modular HPLC, Shimadzu) using a C18 stationary phase (Phenomenex, Gemini; 5 μm, C18, 110 Å, 100 × 10.0 mm) and a gradient of 100 mM aqueous triethylammonium acetate/MeOH. The triethylammonium acetate buffer was set to pH = 8. Oligonucleotide-small molecule conjugates were analyzed by ion pair reverse-phase high-pressure liquid chromatography (HPLC, Prominence Modular HPLC, Shimadzu) using a C18 stationary phase (Phenomenex, Gemini; 5 μm, C18, 110 Å, 100 × 4.6 mm) and a gradient of 10 mM aqueous triethylammonium acetate/MeOH. HPLC traces were recorded at a 254 nm wavelength. For evaporation, the concentrator 5301 from Eppendorf (Wesseling-Berzdorf, Germany) or the RVC 2-18 CDplus from Martin Christ Gefriertrocknungsanlagen GmbH (Osterode am Harz, Germany) was used. Oligonucleotide concentrations were determined by UV spectroscopy using a spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific). Oligonucleotides were analyzed by MALDI-MS (Bruker Daltonics).

METHODS

Affinity Matrix Preparation. The agarose matrix was vortexed and directly transferred into a 1.5 mL Eppi (100 μL, loading of the agarose matrix with N_3 = 15 μmol/mL, 1.5 μmol, 1 equiv, 5 mg). Then, dH$_2$O (650 μL) was added and centrifuged (11,000 rpm, 1 min). A small tip was placed on a large tip, and the solution was pipetted off. After adding dH$_2$O (90 μL), **DNA-1/DNA-2** (10 nmol, 0.07 equiv), and a previously mixed Cu-ligand solution, consisting of CuSO$_4$·5H$_2$O (0.75 μmol, 0.5 equiv), THPTA (3.75 μmol, 5 equiv), and Na-ascorbate (15 μmol, 10 equiv) to the agarose matrix, it was shaken at room temperature (1000 rpm) for 18 h. After the CuAAC-reaction, the matrix was transferred to a filter column. The **capture-DNA-1/DNA-2** was washed three times with 200 μL of dH$_2$O, EDTA (0.1 M), and dH$_2$O.

Affinity Matrix Purification. The **capture-DNA-1** (1.5 mg, 3 nmol of **DNA-1**) was suspended in dH$_2$O (54 μL) and T4-Ligase-buffer (6 μL) and added to the dried reaction mixture, which is to be purified. This mixture was shaken and heated up to 35 °C, kept for 10 min, and then cooled down to 25 °C and kept for 10 min. The mixture was transferred into a filter column and washed three times with 200 μL of dH$_2$O, EDTA (0.1 M), dH$_2$O, DMF, MeOH, MeCN, CH$_2$Cl$_2$, and dH$_2$O each. An aqueous NH$_3$ solution (32%) was used to elute the product **DNA-5**. Therefore, the aqueous NH$_3$ solution (50 μL) was given to the matrix and incubated for 2 min and the

eluate was centrifuged (11,000 rpm, 1 min, 4 °C) and repeated. After this, the matrix was washed with dH$_2$O (2 × 50 μL) and the combined eluates were evaporated at 45 °C. The pellet was re-dissolved in dH$_2$O (40 μL) and analyzed by Nanodrop, analytical HPLC, and MALDI-MS.

Amide Coupling of 1 to DNA-4. To a solution of **DNA-4** (500 pmol) dissolved in 71.3 μL of MOPS-buffer (50 mM, pH = 8, 0.5 M NaCl) was added a mixture of carboxylic acid **1** (2.7 μmol, 5400 equiv) dissolved in 14.3 μL of DMSO (taken from a stock solution: 25 μmol dissolved in 132.7 μL of DMSO), EDC·HCl (1.2 μmol, 2400 equiv) dissolved in 14.3 μL of DMSO (taken from a stock solution: 10.5 μmol dissolved in 125.4 μL of DMSO), HOAt (240 nmol, 480 equiv) dissolved in 14.3 μL of DMSO (taken from a stock solution: 8.4 μmol dissolved in 500 μL of DMSO), and DIPEA (1.2 μmol, 2400 equiv) dissolved in 14.3 μL of DMSO (taken from a stock solution: 10.5 μmol dissolved in 125.4 μL of DMSO), previously activated for 15 min at room temperature. The reaction mixture was shaken at room temperature for 18 h. Afterward, a second aliquot of freshly activated carboxylic acid **1** was added to the reaction mixture and it was shaken for an additional 4 h at room temperature. The amide coupling product **DNA-5** was isolated by either ethanol precipitation or affinity matrix purification.

Ethanol Precipitation. To the reaction mixture was added NaCl (15 μL, 5 M) followed by cold ethanol (400 μL). The samples were kept at −80 °C overnight. Afterward, the sample was centrifuged (11,000 rpm, 50 min, 4 °C). The supernatant was discarded, and the pellet was dried at 37 °C for 10 min. Then, to the pellet was added ethanol (400 μL) and the samples were kept again for 1 h at −80 °C. After centrifugation (11,000 rpm, 50 min, 4 °C), the supernatant was discarded, and the pellet was dried at 37 °C for 10 min. For analysis, the pellet was dissolved in dH$_2$O (40 μL).

ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c02906.

> Analytical data (HPLC, MALDI-MS, UV−Vis), pictures of the purification process, technical drawings of the tailored equipment and experimental procedures (PDF)

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

ADoS, automated dosage system; AIU, automated injection unit; CAD, computer-aided design; CuAAC, copper-catalyzed alkyne-azide cycloaddition reaction; DEL, DNA-encoded library; DIY, do-it-yourself; dsDNA, double-stranded DNA; DSPE, disperse solid phase extraction; FDM, fused deposition modeling; MCU, microcontroller unit; PLA, polylactide; PNA, peptide nucleic acid; SPE, solid-phase extraction; ssDNA, single-stranded DNA

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