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Sample preparation protocols for MALDI-MS of peptides and oligonucleotides using prestructured sample supports

Eckhard Nordhoff[a,*], Martin Schürenberg[b], Gabriela Thiele[c], Christine Lübbert[a], Klaus-Dieter Kloeppel[c], Dorothea Theiss[c], Hans Lehrach[c], Johan Gobom[c]

[a] *Scienion AG, Volmerstr. 7B, 12489 Berlin, Germany*
[b] *Bruker Daltonik, Bremen, Germany*
[c] *Max-Planck-Institute for Molecular Genetics, Berlin, Germany*

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Abstract

This contribution provides a set of protocols for MALDI-MS sample preparation of peptides and oligonucleotides on prestructured sample supports. The protocols have been optimized for high detection sensitivity, robust performance, ease of use, and include sample purification and concentration. Some protocols were optimized for manual preparation of individual samples. Others were developed for the use of automated pipetting stations and optimized for high throughput protein identification and DNA sequence analysis.

Keywords: MALDI; Sample preparation; Proteomics; Oligonucleotide; Peptide

1. Introduction

Today, the most prominent applications of MALDI-TOF-MS in the two growing research fields, proteomics and genomics, focus on protein identification and characterization [1–9], and the detection of DNA sequence variants [10–20], particularly single nucleotide polymorphisms (SNPs) [10–15]. What is analyzed are peptides and oligonucleotides, which in the first instance are the reaction products of a proteolytic digestion of a protein, and in the second instance the products of a Sanger sequencing reaction or one of its modern variants. The aim for high detection sensitivity and sample throughput, both at low costs per sample, dominate current developments and determine the success of MALDI-TOF-MS.

Using state-of-the-art instrumentation, the detection sensitivity is limited by the sample handling and preparation, and not by the mass spectrometric analysis. In MALDI-TOF-MS, the amount of sample consumed per spectrum acquisition and the amount prepared per analysis, can differ by more than six orders of magnitude. One reason for this enormous discrepancy is

* Corresponding author.
E-mail address: nordhoff@scienion.de (E. Nordhoff).

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that only a small portion of the crystalline sample is consumed during MALDI. Typically, the spot size of prepared samples measures 5–15 mm^2 of which only a small fraction (0.002–0.03 mm^2) is laser-irradiated for ion generation.

In addition, the analyte molecules are dispersed throughout the thickness of the sample of which only the top layers are ablated by the hundred or less laser pulses, which are typically used for spectra acquisition. The thin-layer sample preparation technique enhanced analyte utilization by preconcentration of the analyte molecules on the surface of the sample [21].

Regarding sample throughput, heterogeneous crystalline morphology of the prepared samples impairs and slows down automated analysis, and limits the reproducibility of the results. As a consequence, in the worst case instead of many thousand only a few hundred samples can be analyzed per day per instrument. As a consequence, the costs per analysis can be too high, e.g., for the detection of DNA sequence variants.

It has been shown that the problem of sample heterogeneity can be fairly reduced if the sample diameter falls close to the diameter of the laser spot size on the sample support [22,23]. In these studies, preparation of the desired small sample spots was achieved by depositing only a few nanolitres of sample solution, highly enriched in analyte, onto the MALDI support. For routine applications, a disadvantage of this approach is that it requires sophisticated liquid handling technology, and excludes usage of 0.5–20 μL pipette tips, most frequently used for MALDI sample preparation. In addition, to gain maximum detection sensitivity, it is necessary to enrich the sample molecules in a few nanolitres of clean solvent or matrix solution.

To overcome these limitations, a new class of MALDI sample supports (AnchorChip™, Bruker Daltonics, Germany) was developed [24]. These supports provide a prestructured surface consisting of small hydrophilic islands (sample anchors), which measure in diameter 200, 400, 600 or 800 μm, and are surrounded by the strongly solvent-repellent surface of a thin layer of a Teflon-related material. The sample anchors hold the sample droplets, repelled by the surrounding surface, in place and, during solvent evaporation, direct sample deposition onto them. As a result, if the initial matrix concentration is kept low, both sample spot size and sample position are predetermined by the sample support independently of the transferred sample volume. For compatibility reasons, the physical dimensions of the support and the positioning of the sample anchors on the support surface follow the microtitre plate specifications.

Together, above features simplify automated, high throughput MALDI-MS sample preparation and analyses [25]. Because the sample is considerably enriched on the support before matrix crystallization commences, the detection sensitivity is improved provided that the sample is pure. If not, the result can be the opposite because also the contaminants are concentrated. It was demonstrated that, compared to conventional supports, on prestructured sample supports the detection sensitivity for peptides using 2,5-dihydroxybenzoic acid as matrix, and for nucleic acids using 3-hydroxypicolinic acid as matrix, can be improved by a factor of 5–10 if established purification methods are used [24]. For the water-insoluble peptide matrix α-cyano-4-hydroxycinamic acid (CHCA), however, the dried-droplet sample preparation technique, used in these studies, failed under all conditions tested. Here we provide two complementary protocols that overcome this limitation and suggest CHCA as the preferred matrix for peptide analysis on prestructured sample supports.

The logical consequence of the ability to efficiently concentrate the analyte solution on the sample support, is to also integrate sample purification in the procedure. For peptides, this has been achieved by the CHCA affinity sample preparation technique [26]. Each sample anchor is covered with a thin, microcrystalline layer of CHCA, on top of which a droplet of 0.5–2 μL of acidified peptide solution is placed. The peptides in the sample droplet bind to the surface of the CHCA crystals, while salts, buffer components, detergents and chaotropic reagents such as urea or guanidinium hydrochloride remain in solution. In contrast to the thin-layer preparation technique, the sample is not allowed to dry on the support. Instead,

the droplet is removed after 1–3 min incubation time. This technique is easy to use, requires little time, and yields high detection sensitivity and homogeneous samples, which can be analyzed automatically in short time [27]. Using the latest generation TOF-MS/MS instruments, this also accounts for the acquisition of fragment-ion fingerprints.

This paper provides a set of sample preparation protocols optimized for the analysis of peptides and oligonucleotides. In contrast to previous reports [28,29], these protocols were developed for the use of prestructured sample supports. The protocols include sample purification and specific instructions for both, manual and automated sample preparation. For the analysis of peptides, a detailed protocol for matrix-affinity sample preparation is provided which, besides CHCA, now also includes the matrices 2,4,6-trihydroxyacetophenone, sinapic acid, and caffeic acid. For oligonucleotides, the purification is performed within the pipette tip used for sample transfer. For each protocol, a complete list of the suppliers of the used materials and examples are provided as well as known pitfalls and how to avoid them.

2. Experimental

2.1. Instrumentation

- MALDI-TOF mass spectra were recorded on a Bruker Scout MTP Reflex III and Autoflex mass spectrometer (Bruker Daltonics, Bremen, Germany).
- A 96-needle pipetting station, CyBi well (Cy-Bio, Germany).
- A 96-needle pipetting station, MiniTrack V (Packard Bioscience, USA).
- Ultrasound bath (2510, 2.8 L, Branson, USA, or via Novodirect, Germany).

2.2. Materials

- 384/400-μm AnchorChip™ MALDI sample support (Bruker Daltonics, Germany).
- 384/600 μm AnchorChip™ MALDI sample support (Bruker Daltonics, Germany).
- 384/800 μm AnchorChip™ MALDI sample support (Bruker Daltonics, Germany).
- 1536/400-μm AnchorChip™ MALDI sample support (Bruker Daltonics, Germany).
- GELoader tips (Eppendorf, Germany).
- Poly Prep Chromatography Columns (made of polypropylene, Biorad, Germany).
- Stirring rods, 100 mm (Sarstedt, Germany).
- ZipTips (C-18, 0.2-μL bed volume, Millipore, Germany).

2.3. Chemicals

α-Cyano-4-hydroxycinnamic acid (Sigma, Germany).
Acetone (p.a., Merck, Germany).
Acetonitrile (LC-MS Chromasolv, Fluka (Germany).
Ammonium acetate (p.a., Merck, Germany).
Ammonia solution (25% in water, suprapure, Merck, Germany).
Caffeic acid (Sigma, Germany).
Cation exchange resin (AG 50W-X8, 200–400 mesh size, Biorad, Germany).
Diammoniumhydrogene citrate (purity >99%, Fluka, Germany).
2,5-Dihydroxybenzoic acid (Sigma, Germany).
3-Hydroxypicolinic acid (p.a., for mass spectrometry, Fluka, Germany).
Isopropanol (Microselect, Fluka, Germany).
n-Octyl-β-D-glucopyranoside (*n*-OGP) (purity >99%, Fluka, Germany).
n-Hexane (p.a., Merck, Germany).
Sinapic acid (MALDI-MS grade, Fluka, Germany).
Triethylammonium acetate solution (1 M, pH 7.0, HPLC grade, Fluka, Germany).
Trifluoroacetic acid (purity >99.5%, Fluka, Germany).
2,4,6-Trihydroxyacetophenone (>99.5%, p.a., for mass spectrometry, Fluka, Germany).
Ultrapure water (HPLC grade, Merck, Germany).

2.4. Solutions

Solutions containing ammonia	
Ammonia solution	25% ammonia solution/ultrapure water, 20/80 (v/v)
Solutions containing ammonium acetate	
Ammonium acetate solution	2 M ammonium acetate in ultrapure water
Solutions containing diammoniumhydrogene citrate (DAC)	
DAC solution	20 mM in ultrapure water
Solutions for ZipTip purification	
Isopropanol solution (I)	Isopropanol/ultrapure water, 50/50 (v/v)
Isopropanol solution (II)	Isopropanol/ultrapure water, 30/50 (v/v)
Solutions containing triethylammonium acetate (TEAA)	
TEAA solution (I)	1 M in ultrapure water, pH 7.0
TEAA solution (II)	200 mM in ultrapure water, pH 7.0
Solutions containing trifluoroacetic acid (TFA)	
TFA solution (I)	Ultrapure water/TFA, 99.9/0.1 (v/v)
TFA solution (II)	Ultrapure water/TFA, 99/1 (v/v)
TFA solution (III)	Ultrapure water/TFA, 98/2 (v/v)
TFA solution (IV)	40 mM *n*-octyl-β-D-glucopyranoside in TFA solution (III)
TFA solution (V)	Ethanol/acetone/TFA (II) solution, 60/30/10 (v/v/v)
Solutions for the preparation of matrix solutions	
Solution (I)	Acetone/TFA solution (I), 90/10 (v/v)
Solution (II)	Acetone/TFA solution (I), 99/1 (v/v)
Solution (III)	Ethanol/acetone 67/33 (v/v)
Solution (IV)	Acetonitrile/TFA solution (I), 30/70 (v/v)
Solution (V)	Acetonitrile/ultrapure water, 50/50 (v/v)
Solutions for cleaning prestructured sample supports	
Cleaning solution (I)	Acetic acid/isopropanol/ultrapure water, 5/40/55 (v/v/v)
Cleaning solution (II)	0.5 M ammonium acetate in ultrapure water

3. Results and discussion

3.1. MALDI sample preparations for peptides

3.1.1. Matrix solutions

Matrix solutions containing α-cyano-4-hydroxycinnamic acid (CHCA)	
CHCA (I)	Ultrasonicate an excess of CHCA in 500 μL of solution (I) for 30 s and transfer 300 μL of the supernatant to a new vial*
CHCA (II)	Ultrasonicate an excess of CHCA in 200 μL of solution (II) for 30 s, transfer 50 μL of the supernatant to a new vial and add 150 μL of solution (II)*
CHCA (III)	1.6 mM CHCA in solution (III)

Matrix solutions containing caffeic acid (CA)	
CA	Ultrasonicate an excess of CA in 200 μL of solution (II) for 30 s, transfer 100 μL of the supernatant to a new vial and add 100 μL of solution (II)*
Matrix solutions containing 2,5-dihydroxybenzoic acid (DHB)	
DHB	30 mM DHB in solution (IV)
Matrix solutions containing sinapic acid (SA)	
SA (I)	Ultrasonicate an excess of SA in 500 μL of solution (I) for 30 s and transfer the supernatant to a new vial*
SA (II)	Ultrasonicate an excess of SA in 200 μL of solution (II) for 30 s, transfer 50 μL of the supernatant to a new vial and add 150 μL of solution (II)*
Matrix solutions containing 2,4,6-trihydroxyacetophenone (THAP)	
THAP (I)	Ultrasonicate an excess of THAP in 250 μL of solution (II) for 30 s, transfer 150 μL of the supernatant to a new vial and add 150 μL of solution (II)*
THAP (II)	Transfer 50 μL of THAP (I) solution to a new vial and add 50 μL of solution (II)

* The considerably higher saturation concentration of the matrix compound in acetone can take several hours to reach at room temperature. Therefore, to maintain the correct concentration, transfer the supernatant to a new sample vial shortly after ultrasonication. This procedure avoids weighing of matrix aliquots and yields sufficiently accurate concentrations.

3.1.2. Protocol 1: Dried-droplet sample preparation using DHB

1. Deposit 1 μL of clean sample solution on a 400-μm sample anchor.
2. Add 0.5–1 μL DHB solution.
3. Let the sample dry at ambient temperature.

3.1.2.1. Pitfalls and notes. The dried-droplet sample preparation on prestructured sample supports using DHB as the matrix provides efficient analyte enrichment compared to preparations on stainless steel supports, as documented in Fig. 1. Because the high water solubility of DHB excludes on-target washing, the sample has to be purified before. A straightforward approach is ZipTip reversed phase purification following the instructions provided by the manufacturer with the modification that the purified sample is eluted directly onto an aliquot of crystalline DHB, in forehand prepared on the sample anchor. The amount of matrix solution added determines whether the sample anchor is densely covered with DHB crystals or not. For automated spectrum acquisition it is beneficial to completely cover this area with sample. If too much sample is loaded, however, signal resolution and mass accuracy decline. In order to optimize the amount of matrix solution added, we recommend to perform test experiments and to inspect the prepared samples under an optical microscope. Fig. 1C provides a reference for how the samples should look like.

3.1.3. Protocol 2: Dried-droplet sample preparation using CHCA

1. Deposit 0.5 μL peptide sample on a 600-μm sample anchor.
2. Add 2 μL CHCA (III) solution.
3. Let the sample dry at ambient temperature.
4. Wash the sample by adding 5–10 μL TFA (II) solution.
5. Incubate for 30 s, and remove the liquid with a pipette.
6. Recrystallize the MALDI sample by adding 0.7 μL of TFA solution (V).
7. Let the sample dry at ambient temperature.

3.1.3.1. Pitfalls and notes. The ratio of water/organic solvent is critical for the success of the dried-droplet sample preparation on prestructured sample

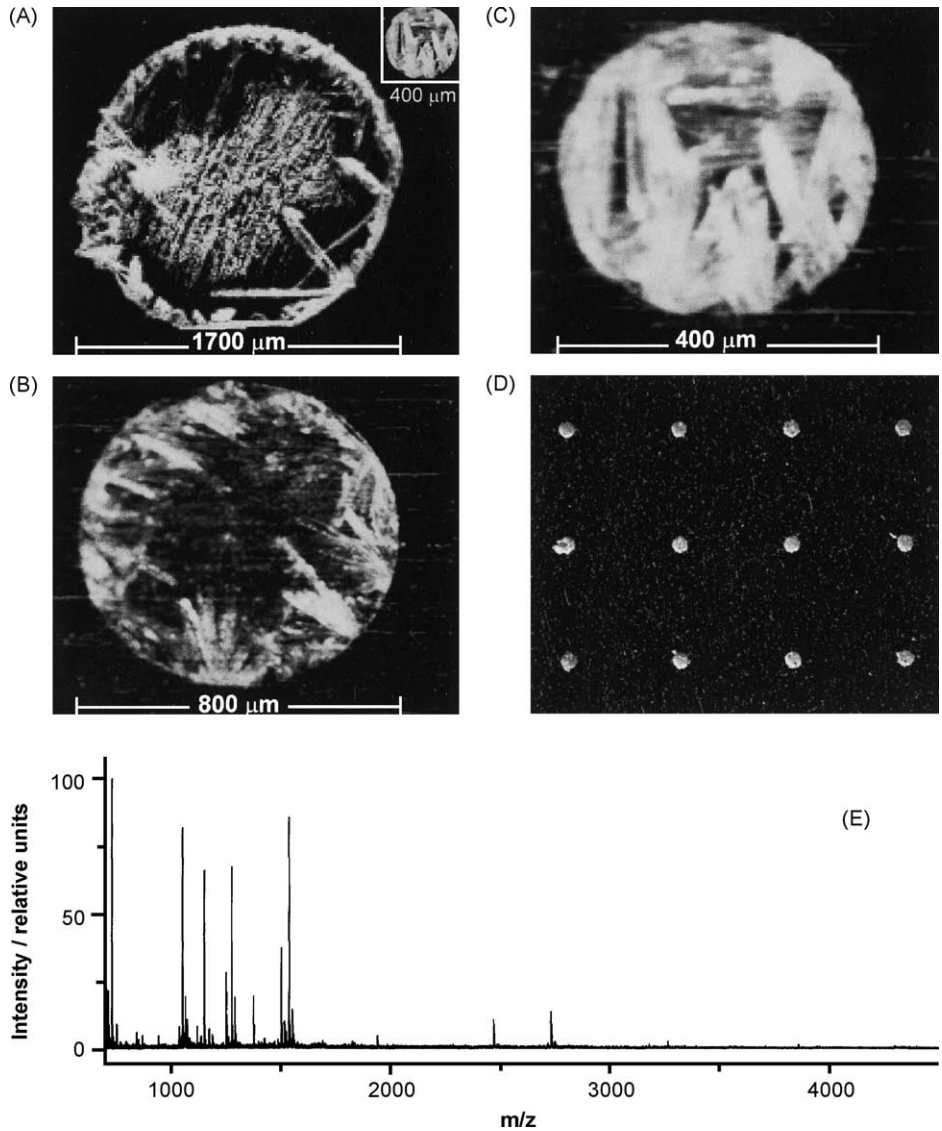

Fig. 1. Dried-droplet sample preparation using 2,5-dihydroxbenzoic acid: (A) sample prepared on an unmodified stainless steel sample support; 0.5 μL of a clean peptide solution and 0.5 μL of a 100 mM matrix solution were mixed on the support. (B) Preparation on an 800-μm and (C) on a 400-μm sample anchor according to Protocol 1. For a direct comparison, the inset in (A) shows the picture shown in (C) reduced in size accordingly. (D) Section of sample array prepared on a 1536/400-μm AnchorChip™ sample support. (E) Mass spectrum of a tryptic digest of human recombinant peptidyl–prolyl *cis–trans* isomerase prepared on a 400-μm sample anchor.

supports when water-insoluble MALDI matrices such as CHCA are used. If the water content in the analyte/matrix solution is too high, matrix precipitation commences before the sample droplet has shrunk to the size of the anchor, with the result that the matrix crystallizes outside the sample anchor. If, on the other hand, too little water is contained in the analyte/matrix droplet, crystallization is confined to the anchor, but the resulting microcrystalline matrix film yields poor mass spectra. To obtain the optimal ratio

of water/organic solvent is difficult in practice, especially for analytes in aqueous solutions for which the resulting water content of the analyte/matrix mixture typically becomes too high. This problem can be circumvented by recrystallizing the dried sample in a suitable solvent. Water-soluble sample components that impair MALDI such as buffer salts, can be washed away prior to the recrystallization step.

The different steps of the protocol are illustrated by micrographs for a clean sample in Fig. 2, and for a contaminated sample in Fig. 3A–F. In the latter case, 0.5 μL of a tryptic digest of bovine serum albumin

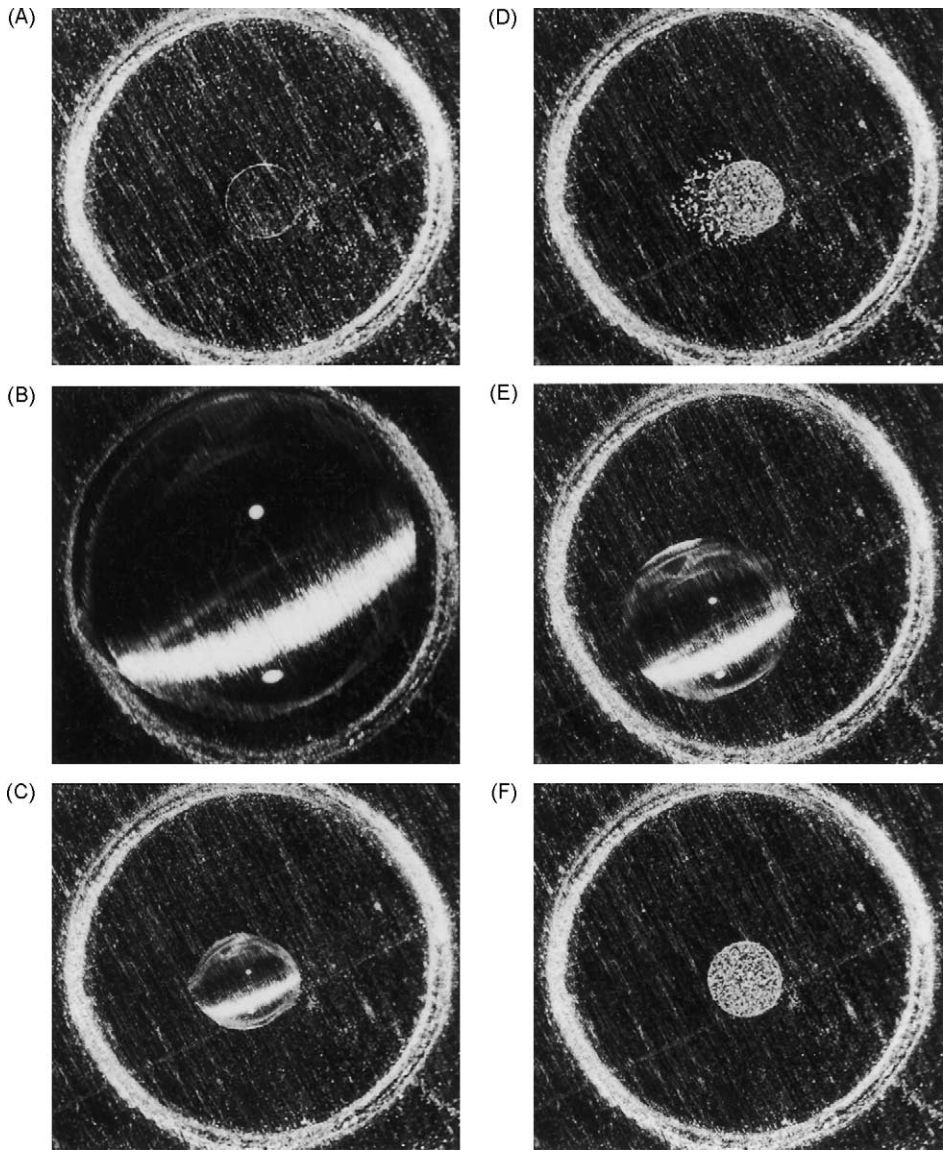

Fig. 2. Dried-droplet sample preparation using α-cyano-4-hydroxycinnamic acid; 600-μm sample anchor (A) before and (B) after deposition of 0.5 μL sample and 2.0 μL matrix solution. (C) As the solvent evaporates the sample is concentrated onto the anchor. (D) The analyte/matrix mixture crystallized partially outside the sample anchor. (E) For recrystallization the dried sample is redissolved in 0.7 μL TFA solution (V). (F) After solvent evaporation the crystalline sample is confined to the sample anchor.

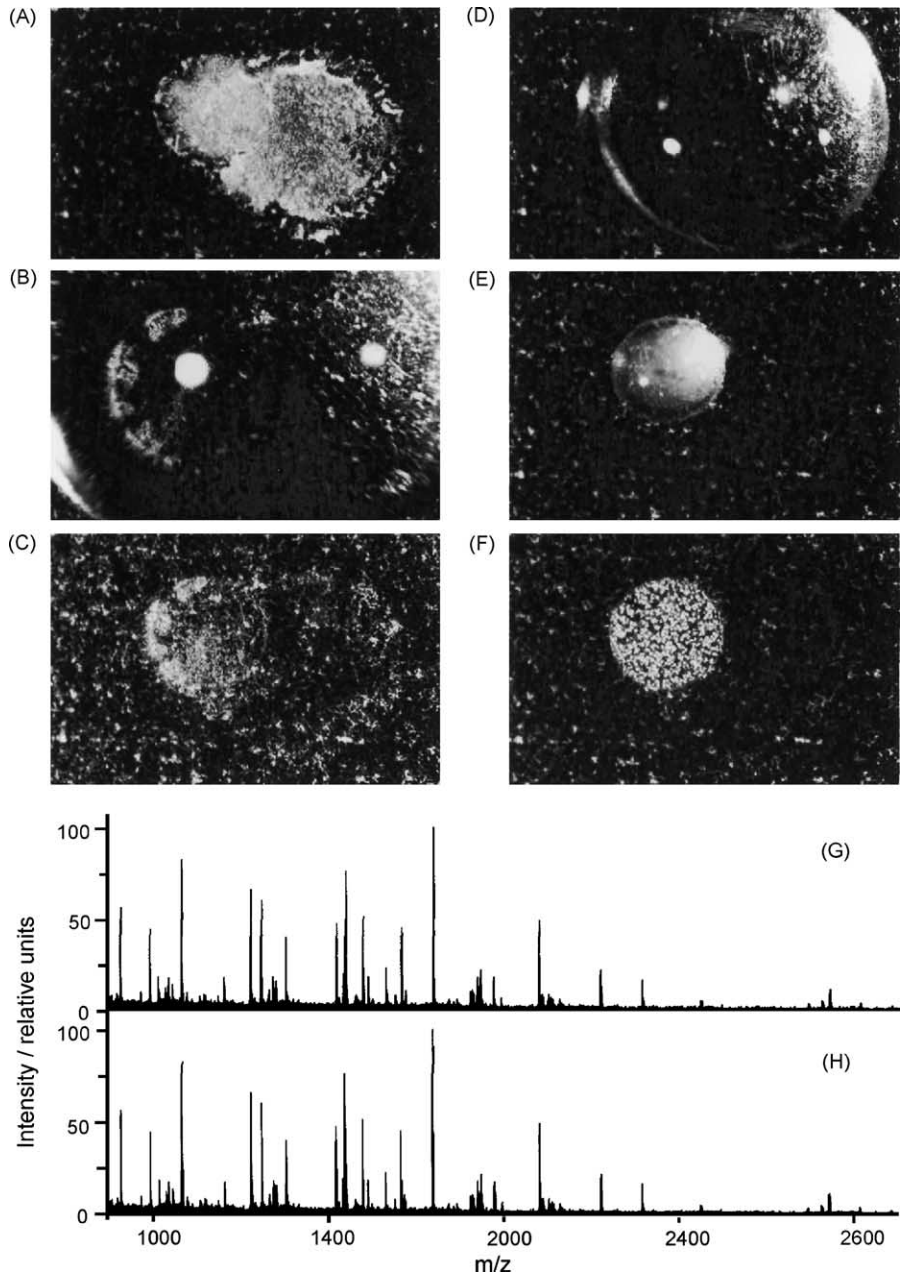

Fig. 3. Dried-droplet preparation of a tryptic digest of bovine serum albumin containing 50 mM ammonium bicarbonate, using α-cyano-4-hydroxycinnamic acid. (A) Crystalline sample after preparation according to the procedure shown in Fig. 2A–D. The sample crystallized partially outside the anchor and salt crystals are visible in the preparation. (B) To remove the contaminants, the sample is covered by a 6 μL droplet of TFA solution (II). (C) After removal of the washing solution, salt has visibly been removed from the sample. (D) The crystalline sample is redissolved in 0.7 μL TFA solution (V). (E) As the solvent evaporates the sample is concentrated onto the anchor. (F) After recrystallization, the sample is confined to the sample anchor. (G) Mass spectrum recorded from the sample. (H) For comparison, the mass spectrum recorded from a second aliquot of the same sample which, instead of washing on the target, was purified before using a ZipTip C-18 pipetting tip and following the instructions provided by the manufacturer.

(2 fmol/μL) dissolved in 50 mM ammonium bicarbonate was deposited on the hydrophilic sample anchor, followed by 2 μL of CHCA (III) solution. Following solvent evaporation, part of the analyte/matrix mixture resided outside the anchor, and salt crystals were present in the sample (Fig. 2A). Five microlitre TFA (I) solution were added and incubated with the sample for 30 s (Fig. 2B). After aspirating the washing solution, a large part of the salt had visibly been removed (Fig. 2C); 0.7 μL of solution (IV) were then added to completely redissolve the sample (Fig. 2D). Recrystallization commenced when the sample droplet had shrunk to the size of the anchor (Fig. 2E), resulting after complete solvent evaporation in a sample confined to the anchor (Fig. 2F).

3.1.4. Protocol 3: Matrix-affinity sample preparation

3.1.4.1. Sample preparation on all positions of the sample support.

1. Deposit 150 μL of matrix solution (CHCA (I), SA (I), THAP (I), or CA) near one of the short edges of a 384/400-μm AnchorChip™ sample support.
2. Place a stirring rod horizontally across the sample plate surface, contacting the matrix solution, which thereby spreads out across the length of the rod, creating a liquid film between the rod and the sample support.
3. Draw the rod across the sample support, moving the matrix liquid film along, such that a small volume of matrix solution adheres to each sample anchor (Fig. 4A). The solvent quickly evaporates and leaves behind a homogeneous layer of crystalline matrix.
4. To each peptide sample, add TFA (III) solution to a final concentration of 0.25% TFA (v/v) and 5 mM *n*-OGP (Fig. 4B).
5. Deposit 0.5–2 μL of each sample onto one matrix spot.
6. After 1–3 min incubation remove most of the remaining sample solution by absorption using soft paper tissue or by aspiration using a pipetting station (Fig. 4C).
7. Wash the samples by dispensing 3 μL of TFA (I) solution onto each sample spot. After 5 s remove most of the solution as described above (step 6).

3.1.4.2. Preparation of samples on only a few positions of the sample support.

1. Immerse a GELoader pipette tip in the matrix solution (CHCA (II), SA (II), THAP (II), or CA) to aspirate a small volume by capillary force.
2. Touch each sample anchor briefly with the pipette tip to deposit a small volume of the matrix solution (Fig. 4D).
3. To each peptide sample, add TFA (III) solution to a final concentration of 0.25% TFA (v/v) and 5 mM *n*-OGP.
4. Deposit 0.5–2 μL of each sample onto the matrix layers (Fig. 4E).
5. After 1–3 min incubation, remove the remaining sample solution by absorption using soft paper tissue (Fig. 4F), or by aspiration using a manual pipette.
6. Wash the dried samples by adding 2 μL of TFA (I) solution.
7. After 10 s remove the washing solution as described above (step 5).

3.1.4.3. Pitfalls and notes. The matrix-affinity sample preparation integrates analyte purification and enrichment. Peptide molecules bind to the crystalline matrix layer during a short incubation time, after which the remaining sample liquid is removed, and the sample washed. The preparation therefore tolerates high concentrations of many water-soluble sample components, including phosphate salts. The simplicity of the preparation allows efficient parallel sample preparation, e.g., by simultaneous preparation of 96 or 384 samples contained in a microtitre plate using a pipetting station. The MALDI samples are very homogeneous, and little variation of the laser power is needed for different samples, thereby facilitating automatic spectra acquisition. The flat topology of the samples results in improved resolution and mass accuracy for MALDI-TOF-MS analysis. The addition of *n*-OGP to the samples fills the dual function

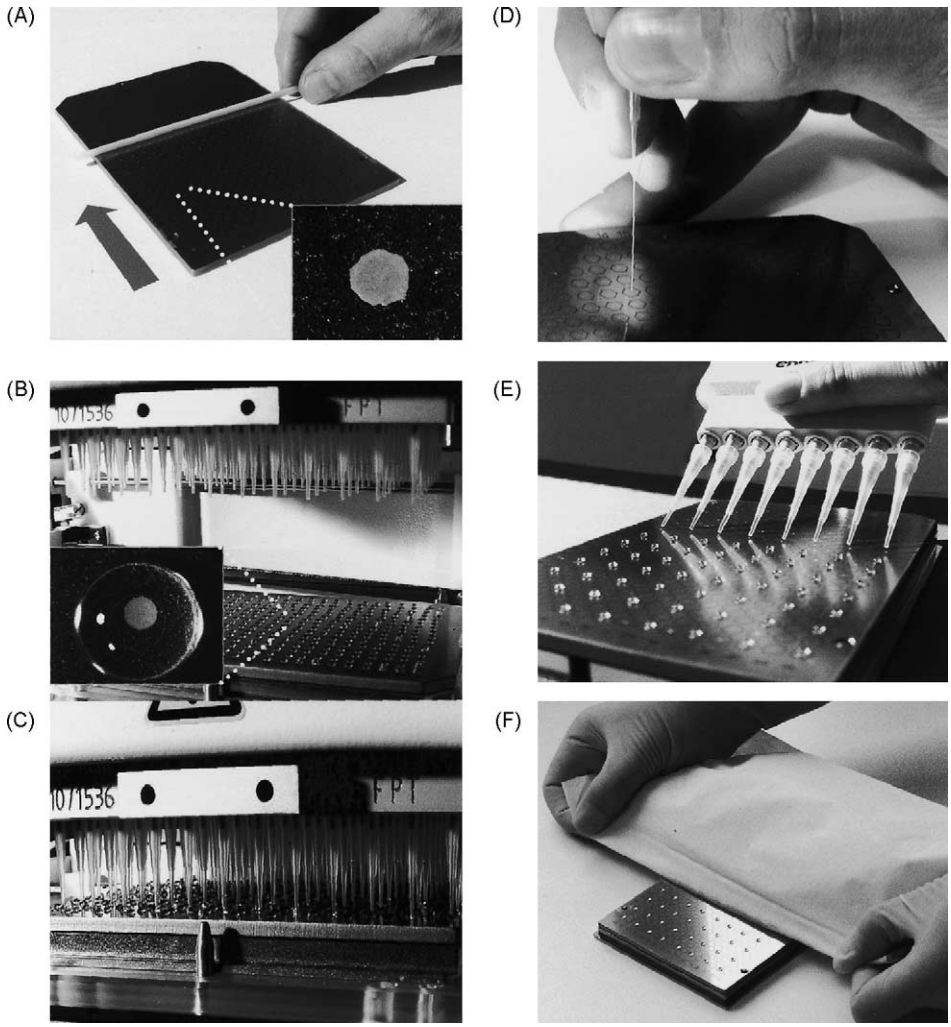

Fig. 4. Matrix-affinity sample preparation. (A–C) Preparation of many samples: (A) the matrix solution is spread across the prestructured sample support. Inset: magnification of a matrix spot generated by the procedure. (B) Samples are transferred from a 384 microtitre plate using a 96-needle pipetting station. Inset: magnification of a sample droplet on a matrix spot. (C) After incubation, most of the sample liquid is removed by the pipetting station. (D–F) Manual sample preparation: (D) matrix solution is deposited on single sample plate positions using a GELoader pipette tip. (E) Samples are deposited onto the matrix spots using a manual pipette. (F) After 1–3 min incubation, the remaining liquid is removed by absorption using soft paper tissue.

of enhancing the solubility of the peptides, thereby reducing losses due to adsorption to sample vials and pipette tips, and to minimize oxidation of methionine and tryptophane residues, which is otherwise frequently observed with this preparation technique.

The amount of matrix deposited on the sample anchors is critical for good results. If too much matrix is deposited, the resulting crystalline layer is inhomogeneous and yields poor spectra. If, on the other hand, too little matrix is deposited, the matrix layer becomes too thin and is dissolved by the sample solution. When applying the matrix by spreading the matrix solution over the sample support, the amount of deposited matrix can be controlled, either by modifying

the concentration of the matrix compound or by varying the water concentration of the solution. Higher water concentration leads to adherence of a larger volume to the hydrophilic anchors and lower concentrations reduce it. The optimal parameters may vary slightly depending on the quality of the sample support surface. Therefore, it is useful to inspect a few of the prepared matrix spots under a microscope. The matrix should form a dense, homogeneous microcrystalline film, such as the one shown in the inset of Fig. 4A. Matrix spots prepared using a pipette tip (Fig. 4D) should have the same appearance.

Matrix-affinity sample preparations tolerate relatively high amounts of sample components that impair MALDI, e.g., phosphate, sodium, and potassium salts. The sample must, however, not contain organic solvents, since these dissolve the matrix layer. For the same reason, the sample must be acidified before deposition onto the matrix layer. For this purpose TFA can be used up to a concentration of approximately 1% v/v. Using weaker organic acids, such as formic acid or acetic acid, for which a higher concentration (5–10%, v/v) may be required to acidify the sample, is not recommended because they can dissolve the matrix layer.

The matrix-affinity sample preparation was developed for the matrix CHCA, but was found to also be usable with other matrix compounds of low water-solubility, especially sinapic acid (SA), 2,4,6-trihydroxyacetophenone (THAP) and caffeic acid (CA). For comparison, Fig. 5 shows four mass spectra which were recorded from four equal aliquots of a tryptic digest of human recombinant peptidyl–prolyl *cis–trans* isomerase using these matrices (Fig. 5B–D) and, as reference, CHCA (Fig. 5A). The comparison reveals that, in the shown example, the performance of the different matrices is complementary, which in return suggests the use of more than one matrix for MALDI-TOF-MS peptide mapping [28]. For instance, in the shown example the larger cleavage products yielded stronger signals with THAP and CA than with CHCA or SA, and the smaller peptides were more efficiently detected when using SA or THAP, instead of CHCA or CA.

3.2. MALDI sample preparations for oligonucleotides

3.2.1. Matrix solutions

Matrix solutions containing 3-hydroxypicolinic acid (HPA)	
HPA (I)	250 mM HPA in solution (V)
HPA (II)	100 μL HPA (I) + 320 μL ultrapure water + 160 μL isopropanol + 50 μL cation-exchange resin suspension (50% resin, w/w) + 17 μL of DAC solution. Add the cation exchange resin minimum 30 min before using the matrix solution. For preparation of the cation exchange resin see Protocol 4

3.2.2. Protocol 4: Preparation of cation exchange resin for oligonucleotide analysis

1. Fill 6 g cation exchange resin into a chromatography column.
2. Wash three times with solution (V).
3. Wash two times with 10 mL ammonia solution (5%).
4. Wash three times with 10 mL ammonium acetate solution.
5. Wash three times with ultrapure water.
6. Divide the resin into 100 μL portions (suspension, 50% beads v/v) and store at 4 °C.

3.2.2.1. Pitfalls and notes. The protocol ensures efficient loading of the resin with ammonium ions. The resin should form a homogeneously packed column and never run dry during operation. Step 2 is necessary to remove soluble organic compounds which otherwise will contaminate the HPA (II) matrix solution. It is very important to avoid any contamination with metal cations during this preparation. Alkali ions are ubiquitous (glassware, dust particles) and, therefore, it is recommended to use only highly pure chemicals and to perform this preparation without glassware

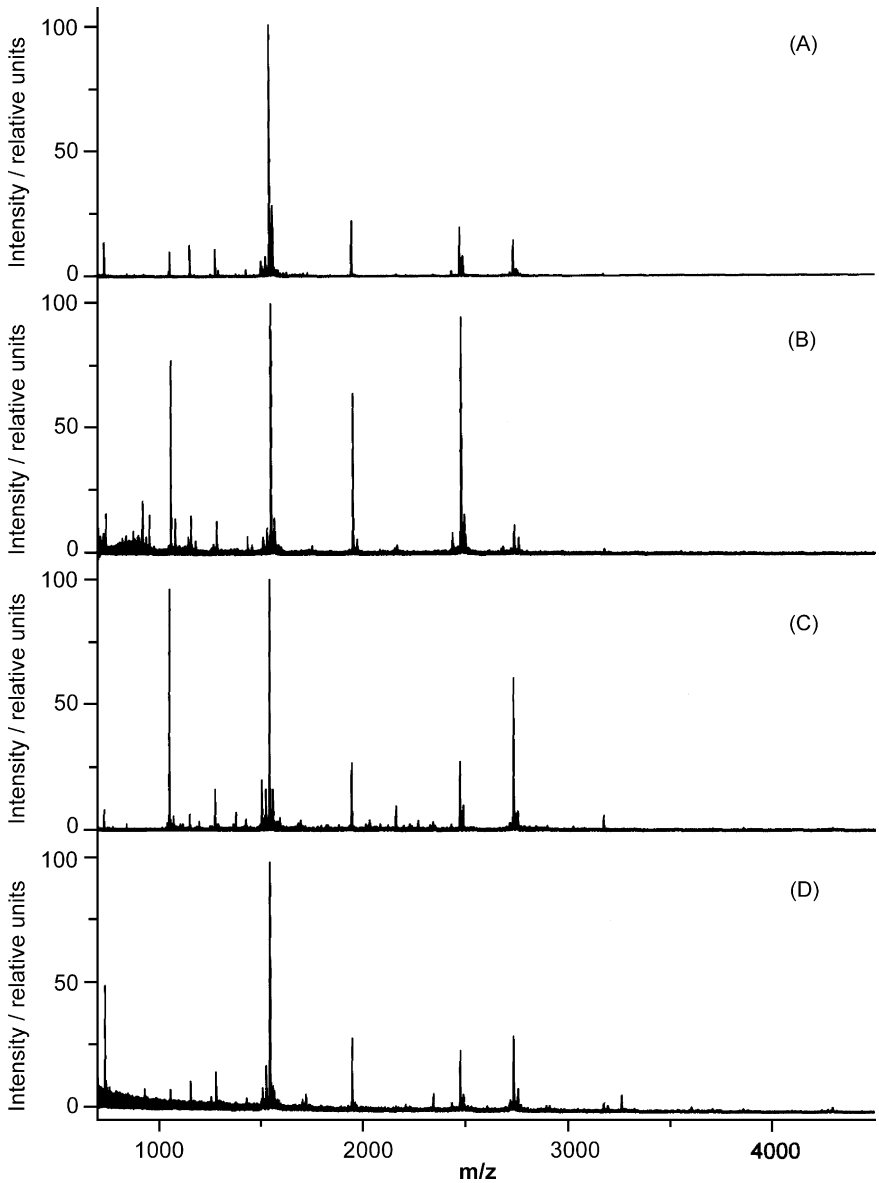

Fig. 5. Mass spectra of a tryptic digest of human recombinant peptidyl–prolyl *cis–trans* isomerase. The samples were prepared on 400-μm sample anchors by the matrix-affinity method, using (A) CHCA, (B) SA, (C) THAP, and (D) CA as matrix compounds.

and in a dust-free environment. The storage at 4 °C of the prepared resin should not exceed 3 months and a fresh fraction of the resin should be used every day because frequent pipetting of small aliquots is likely to contaminate the resin with dust particles, which contain alkali ions or other metal cations.

3.2.3. Protocol 5: Sample preparation of purified oligonucleotides

3.2.3.1. Purified, metal cation-free samples.

1. Deposit 1.0 μL of HPA (II) solution on a 400 μm sample anchor.

2. Let the matrix crystallize on the anchor at ambient temperature.
3. Add 1.0 μL of the sample solution.
4. Let the sample dry at ambient temperature.

3.2.3.2. Purified samples which contain small amounts of metal cations.

1. To an aliquot of 5 μL sample solution add 2 μL of cation exchange resin.
2. Incubate for 30 min at room temperature or shake for 10 min.
3. Deposit 1.0 μL of HPA (II) solution on a 400 μm sample anchor.
4. Let the matrix crystallize on the anchor at ambient temperature.
5. Add 1.0 μL of the sample solution (supernatant).
6. Let the sample dry at ambient temperature.

3.2.3.3. Pitfalls and notes. Protocol 5 (Subsection 3.2.3.1) requires very pure, metal cation-free samples. If the samples contain small amounts of metal cations (<1 mM), these are quantitatively exchanged by ammonium ions in the steps 1 and 2 in Protocol 5 (Subsection 3.2.3.2). Examples for this are shown in Fig. 6. Whether a sample is considered pure or contaminated, naturally, depends on the requirements of the next experiment or the analysis technique applied. For MALDI-MS analysis of oligonucleotides, small amounts of peptides or proteins (less than oligonucleotide) can usually be tolerated because, compared to CHCA, SA, CA, DHB or THAP, the matrix HPA is little efficient for the desorption/ionization of peptides and proteins. Ammonium salts present in the sample (e.g., ammonium acetate or diammonium citrate) can even improve the analysis of oligonucleotides if their molar quantities, compared to the amount of HPA, is small (<5%). Most critical are metal cations such as Na^+, K^+, Mg^{2+}, or Ca^{2+}, detergents, especially ionic (e.g., SDS) and neutral, high molecular weight compounds (e.g., NP40 or any member of the Triton or Tween series, commonly used in molecular biological laboratories), and little volatile liquid additives such as glycerol or formamide. Whereas the latter two categories of contaminants inhibit sample crystallization, the former result in multiple molecular ion species for each oligonucleotide (Fig. 6B), thereby degrading the detection sensitivity and rendering data interpretation for mixture analyses difficult.

3.2.4. Protocol 6: ZipTip purification

1. Deposit 1.0 μL of HPA (II) solution on a 400 μm sample anchor.
2. Let the matrix crystallize on the anchor at ambient temperature.
3. To an aliquot of 1–10 μL sample solution add 3 μL TEAA solution (I).
4. Wet the ZipTip (C-18, 0.2 μL bed volume) by aspirating and dispensing to waste three times 10 μL of isopropanol solution (I).
5. Condition the ZipTip by aspirating and dispensing to waste three times 10 μL TEAA solution (II).
6. Slowly aspirate and dispense the total sample solution 10 times.
7. Wash the bound sample by aspirating and dispensing to waste five times 10 μL of TEAA solution (II).
8. Elute the sample with 1.5 μL of isopropanol solution (II) directly onto the crystalline matrix spot (Fig. 7).

3.2.4.1. Pitfalls and notes. Although C-18 particles are immobilized in the outlet of tips, the mode of binding and elution, using TEAA as ion-pair reagent, differs from the conditions known for reversed phase HPLC purification of oligonucleotides. Particularly, a considerably higher percentage of organic solvent (30–40% acetonitrile or 20–30% isopropanol instead of 20% acetonitrile) is often required to quantitatively elute bound oligonucleotides in a small volume (<2 μL). It appears that the plastic polymer matrix used to immobilize the chromatography resin, alters the elution conditions. Typical yields are 40–50% if more than 5 pmol and less than 50 pmol of oligonucleotide are contained in 10 μL sample volume. Binding of the sample requires time and intensive contact of the liquid phase with the stationary phase. Therefore, the protocol includes 10 cycles of aspiration and dispensing (step 6). If the sample concentration is

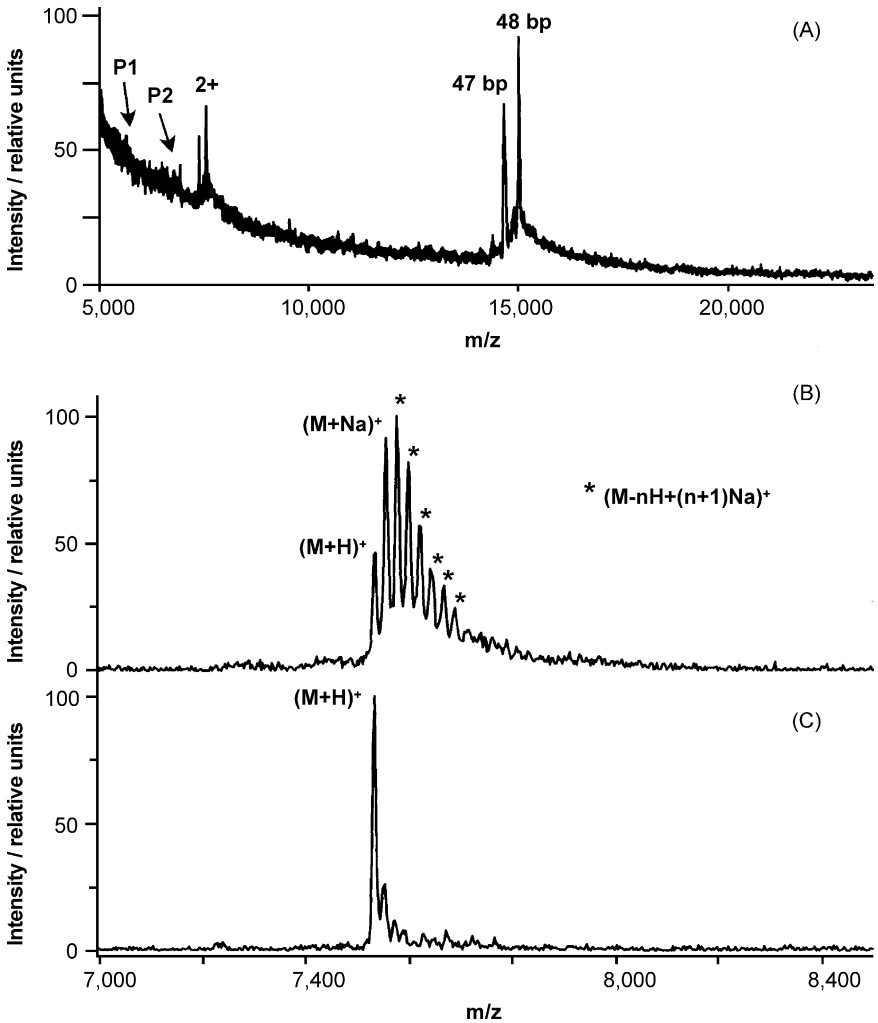

Fig. 6. (A) Mass spectrum of 300 fmol of a 47 bp PCR product of human DNA, which was purified on magnetic particles following the instructions provided (genopure, Bruker Daltonics, Germany). One microlitre of a total of 10 μL purified sample was prepared for analysis on a 400-μm sample anchor according to Protocol 5 (Subsection 3.2.3.1). In addition to the expected product, a known sequence variant differing from the wild type by the insertion of one nucleotide (48 bp PCR product) was detected; 47/48: overlapping signals of the two single strands of the two PCR products. P1, P2: signals of the primer used for amplification. (B, C) Mass spectra of a custom synthesized DNA 25-mer. The C-18-cartridge purified sample received, was prepared for analysis according to (B) Protocol 5 (Subsection 3.2.3.1), omitting and (C) according to Protocol 5 (Subsection 3.2.3.2), including incubation of the sample with cation exchange resin.

high (e.g., 5 μM), however, the number of cycles can be reduced to five or six.

For a new tip, wetting the immobilized resin with a high percentage of organic solvent (e.g., 50% isopropanol, step 4) and subsequent washing with TEAA buffer (step 5) is crucial for the yield of the purification. If the sample is not contaminated with particles, reuse of the tip is possible. A general trend is that the yield of the purification declines with increasing length of the oligonucleotide.

Above 50 nucleotides, performance can be very poor. The latter accounts for double-stranded DNA

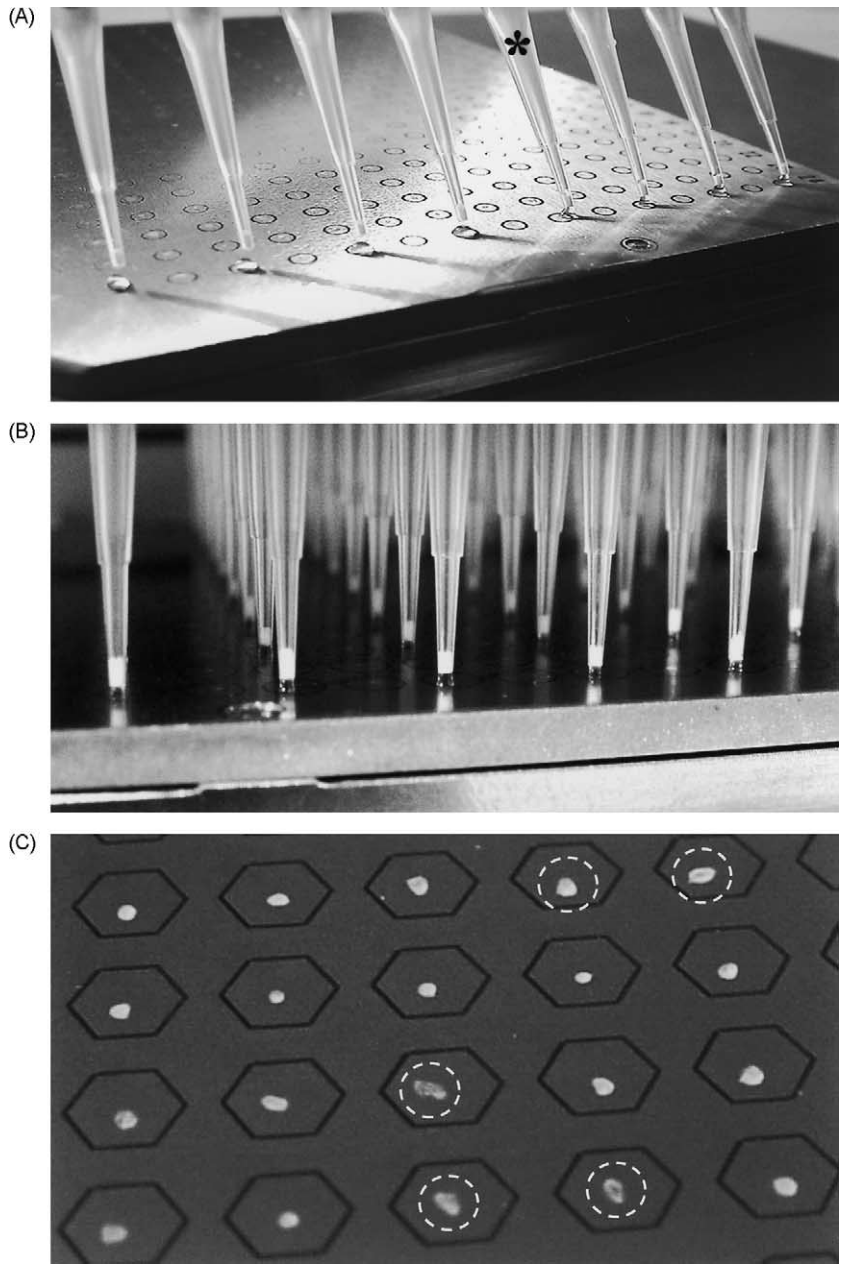

Fig. 7. Sample preparation of oligonucleotides. (A) Manual ZipTip purification. The purified oligonucleotides are eluted onto dry aliquots of the matrix HPA prepared on 400-μm sample anchors. The use of a multichannel pipette is straightforward because the prestructured surface of the support corrects for imperfections of the tip alignment. (∗) Although the tip is not aligned, the sample solution is directed onto the sample anchor. It adheres to the anchor and is repelled by the surrounding surface. (B) High throughput sample preparation using a MiniTrack V (Packard Bioscience, Germany) 96-needle pipetting station. (C) Section of a prepared sample array. Broken circles: part of the sample crystallized outside the sample anchor. This indicates that the surface surrounding these anchors has lost part of its strong hydrophobicity. If the surface is not scratched or chemically damaged, this problem can often be overcome by incubating the sample support in *n*-hexane for 30 min.

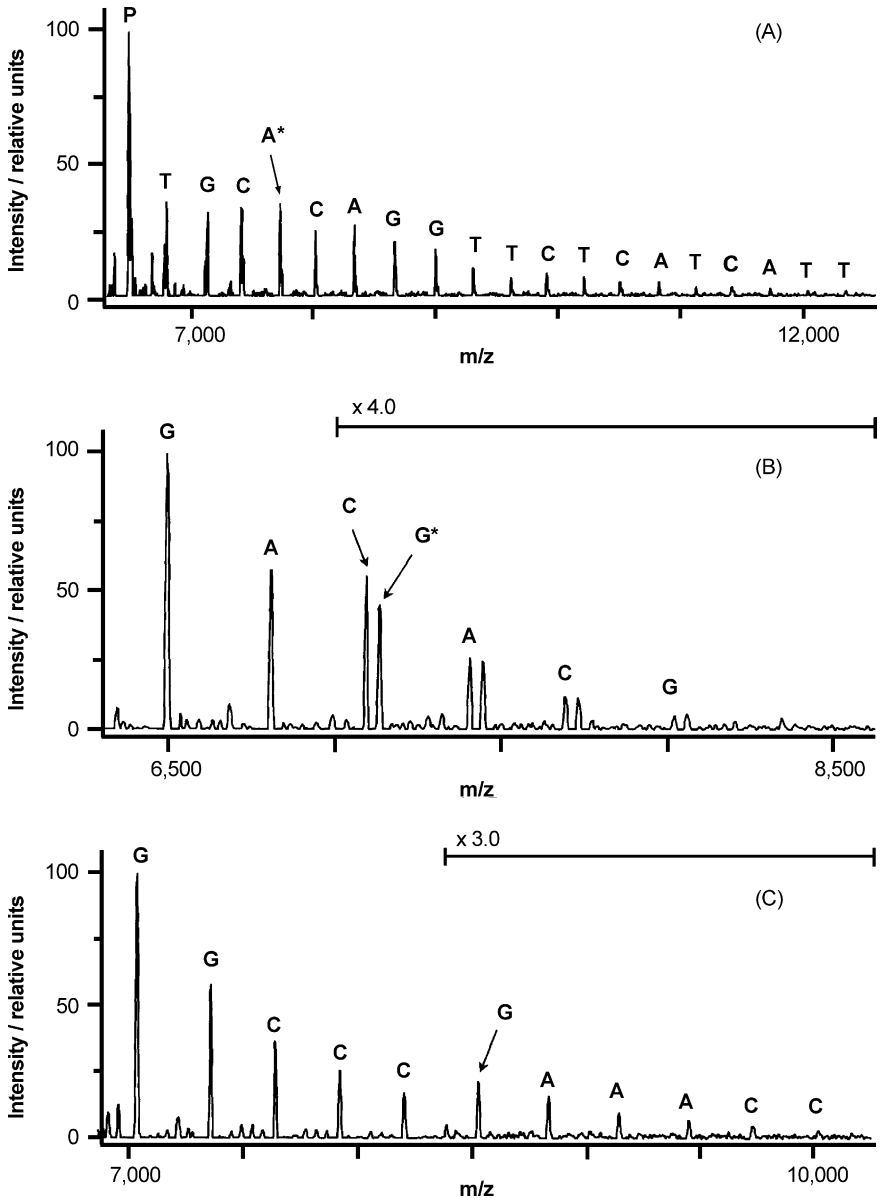

Fig. 8. Partial sequencing of different PCR products of the human cytochrome P450 cyp2d6 gene of different individuals by combining a four-in-one Sanger cycle sequencing reaction with MALDI-TOF-MS. (A) A 220 bp PCR product of exon 6 of an individual was sequence analyzed around the known G/A-polymorphic nucleotide G^{4469} (reverse strand). (B) A 1006 bp PCR product harboring exon 3–5 of an individual was analyzed around the known C/G-polymorphic nucleotide C^{3280} (reverse strand). (C) A 238 bp PCR product of exon 2 of an individual was analyzed. The sequence ladder covers the known G/C-polymorphic nucleotide G^{2658} (reverse strand). Arrow: indicates the expected polymorphic nucleotide, (∗): detected variant nucleotide, P: sequencing primer. All sequencing products were ZipTip purified according to Protocol 6, and subsequently mass analyzed in automated linear acquisition mode.

in general. ZipTip reversed phase purification of oligonucleotides is efficient for removing nucleotide monomers, salts, buffer components, glycerol, and other hydrophilic compounds. To separate peptides, proteins or detergents, however, this technique is not well suited. For this purpose as well as the purification of PCR products (Fig. 6A), we use magnetic particles whose surface has been optimized for reversible binding of single-stranded as well as double-stranded nucleic acids (genopure, Bruker Daltonics, Germany). Three examples that demonstrate the performance of ZipTip reversed phase purification for the analysis of sequencing reaction products, are documented in Fig. 8.

3.3. Reuse of prestructured sample supports

3.3.1. Protocol 7: Cleaning of prestructured sample supports

3.3.1.1. For the analysis of peptides.

1. Separate the target from the holder.
2. Rinse the target with acetone.
3. Rinse the target with cleaning solution (I).
4. Ultrasonicate the target two times for 5 min in a fresh aliquot of cleaning solution (I).
5. Rinse the target with Millipore water.
6. Dry the target in a stream of clean nitrogen or compressed air.

3.3.1.2. For the analysis of oligonucleotides.

1. Separate the target from the holder.
2. Rinse the target extensively with Millipore water.
3. Rinse the target with acetone.
4. Rinse the target with ethanol.
5. Ultrasonicate the target two times for 5 min in a fresh aliquot of cleaning solution (II).
6. Rinse the target with Millipore water.
7. Dry the target in a stream of clean nitrogen or compressed air.

3.3.1.3. Pitfalls and notes. When using organic solvents in an ultrasound bath, consider the safety instructions provided by the hardware manufacturer. To save expensive solvents and aid removal of previous samples from the anchors, assist rinsing the target with a particle-free paper tissue. To avoid scratches, move the paper tissue only gently across the support surface. Before sample preparation, it is recommended to inspect the target carefully by eye, best under a microscope, to ensure that all crystals from previous samples have been removed. Whenever memory effects are likely to occur, e.g., an analysis of picomole amounts of peptides is followed by an analysis in the low fmol range, it is recommended to perform the cleaning procedure twice or, if available, to use a separate sample support for small sample amounts.

If the sample support surface (or parts of it) has lost its strong hydrophobicity (see Fig. 7C), the support can often be reconditioned by 30-min incubation in *n*-hexane, followed by the standard cleaning procedure. The lyophobic coating is fairly inert towards organic solvents and low pH, but is degraded in alkaline solutions. For short times (<1 min), the target may be exposed to weakly alkaline solutions (pH < 9.5), for long time exposure, the solution should be neutral or weakly acidic. Strong ultrasonication and ultrasonication over long periods (>20 min) can also damage the hydrophobic coating. Therefore, mild ultrasonication conditions and short incubations times are recommended.

For oligonucleotides, ultrasonication in 0.5 M ammonium acetate replaces exchangeable metal cations on the target surface by ammonium ions. This is the most severe problem when analyzing nucleic acids, especially if these contain more than 30 nucleotides. For the analysis of peptides, memory effects are the most severe. For this reason, we recommend to strictly use separate targets for peptides and oligonucleotides.

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