



● Automated High-Throughput Oligo QC: From Automated Sample Preparation to MALDI Spectra Assessment

Synthetic oligonucleotides find a variety of applications in molecular biology. Primers for instance are used for DNA sequencing and amplification. Mass spectrometry is a well-established technology for quality control of many synthetic products including oligonucleotides.

Abstract

Here we describe a protocol to prepare typical primers on a robotic platform for MALDI analysis and the subsequent

automated acquisition and interpretation of the mass spectra. MALDI mass spectrometry is ideal for rapid screening and is already established in many high-throughput primary screens

[1,2]. It is much more specific than optical detection methods and avoids the complexity and limited throughput of HPLC.

Keywords:
High-throughput
analysis, MALDI-TOF,
3-HPA, oligonucleotides,
primer, quality control

Introduction

MALDI-TOF mass spectrometry is a robust and easy-to-use approach to check the identity and purity of oligonucleotides even without HPLC purification. However, sample preparation used to depend on technicians' skills and experience which was a limiting factor for widespread adoption. Automated robotic preparation protocols can overcome these obstacles providing reliable and reproducible results while refocusing the scientists time on more value-added tasks.

In case of oligonucleotides like DNA primers, sample preparation requires simple pipetting steps easily

applicable in robotic protocols. To allow automated sample preparation and targeted spectra acquisition from defined sample spots, a dedicated sample holder technology using hydrophilic anchors on a hydrophobic surface was used (MTP AnchorChip 384).

MALDI-TOF MS spectra were acquired and processed automatically. Quality attributes of the primers were analyzed in Biopharma Compass 2021 and described in dedicated reports. The quality control of a sample is automatically passed when the spectrum corresponds to the expected analyte as defined in the workflow method.

Table 1: List of materials used

Material	Order Information/Details
PCR-Plate (96 wells)	Eppendorf twin.tec® PCR Plate 96, # 0030 128.648
MALDI Matrix 3-HPA	3-Hydroxypicolinic acid (Bruker # 8201224)
DAC	Diammonium citrate (Merck #247561)
ACN	Acetonitrile (Biosolve #01204101)
H2O	Deionized Water
MALDI Sample Plate	MTP AnchorChip 384 (800 µm), (Bruker # 8280790)
Automated Liquid Handling Station	Bravo Automated Liquid Handling Platform (Agilent)
BPC	BioPharma Compass 2021 (Bruker)
Oligonucleotide Calibration Standard	Bruker #206200

Table 2: List of universal primer names and sequences used (Kaneka Eurogentec, HPLC purified).

M13 Reverse (-27)	CAG GAA ACA GCT ATG AC
M13 Forward (-20)	GTA AAA CGA CGG CCA GT
pET 3'	CTA GTT ATT GCT CAG CGG
pET 5' (T7)	TAA TAC GAC TCA CTA TAG G
T7 Promoter	TAA TAC GAC TCA CTA TAG GG
Neomycin Rev	AGG TGA GAT GAC AGG AGA TC

Experimental

Liquid handler protocol for automatic MALDI sample preparation:

The robot was equipped with two 96-well PCR plates, one preloaded with [Matrix solution](#) and one with [Sample solutions](#).

- Preparation of DAC solution:** Dissolve 1 mg of DAC in 1000 µL of deionized water, vortex and spin down.
- Preparation of 3-HPA/DAC Matrix solution:** Dissolve 15 mg of 3-HPA in 1000 µL of DAC solution (1 mg/mL), vortex and spin down.
Note: the quality of the Matrix solution is best after a few days storage in the dark at room temperature.
- Sample solution:** Dissolve each oligonucleotide in water to obtain approx. 500 fmol/µL.
- MALDI sample preparation:** Transfer 1 µL of the [Matrix solution](#) onto each MALDI target plate position and allow to dry (~5-10 min drying time).
- Transfer 1 µL of the [Sample solution](#) onto each matrix spot and allow to dry (~5-10 min drying time).
Note: Synthetic oligos should not be dissolved in salt containing solutions (PBS, etc.) prior to this MALDI sample preparation to avoid excessive salt adducts.

Data acquisition:

Automatically prepared oligo samples were analyzed by MALDI-TOF MS in positive linear ion mode on the autoflex maX using the default flexControl method for the mass range from 5 to 20 kDa. The method was calibrated in the central sample spot of the AnchorChip targets (chip_1) using the Bruker Oligonucleotide Calibration Standard prepared in the same way with the 3-HPA/DAC matrix in the mass range 3-9 kDa. During automated acquisition, the laser shots were focused on the matrix crystals at the rim of the anchor spots using the “three circles” raster in the autoXecute method.

Data analysis:

Spectra were automatically processed in the flexAnalysis software using a customized processing method based on the standard method for small proteins (Proteins 5-20kDa_Full_Process.FAMSMETHOD). The mass range was set to 3500-10000 Da and S/N threshold was set to 3. Processed spectra were imported into BioPharma Compass (BPC). For multi attributes screening analysis of the primers, the BPC workflow Multi Target Screening MALDI was used and dedicated acceptance criteria were defined (Figure 3). The dominant MH⁺ ion was used for confirmatory purposes – higher charge states were not included in the analysis.

Results

Six different universal primers (Table 2) in the range between 17-mer to 20-mer were measured in 8 replicates each and analyzed in BPC using the workflow Multi Target Screening MALDI. This workflow requires a Sample Table providing sample

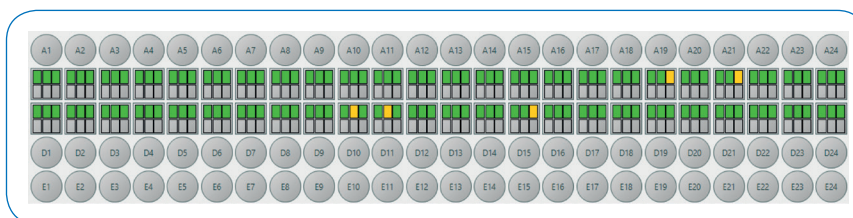


Figure 1: Status overview of analyzed MALDI spectra in Biopharma Compass 2021, displaying the status of 3 quality attributes for 48 sample spots. The traffic light report of these quality attributes reflects the success of the analysis vs. expected results to minimize the need for manual data validation. A detailed report of the quality attributes of the selected position C12 results and a legend is shown in Figure 4.

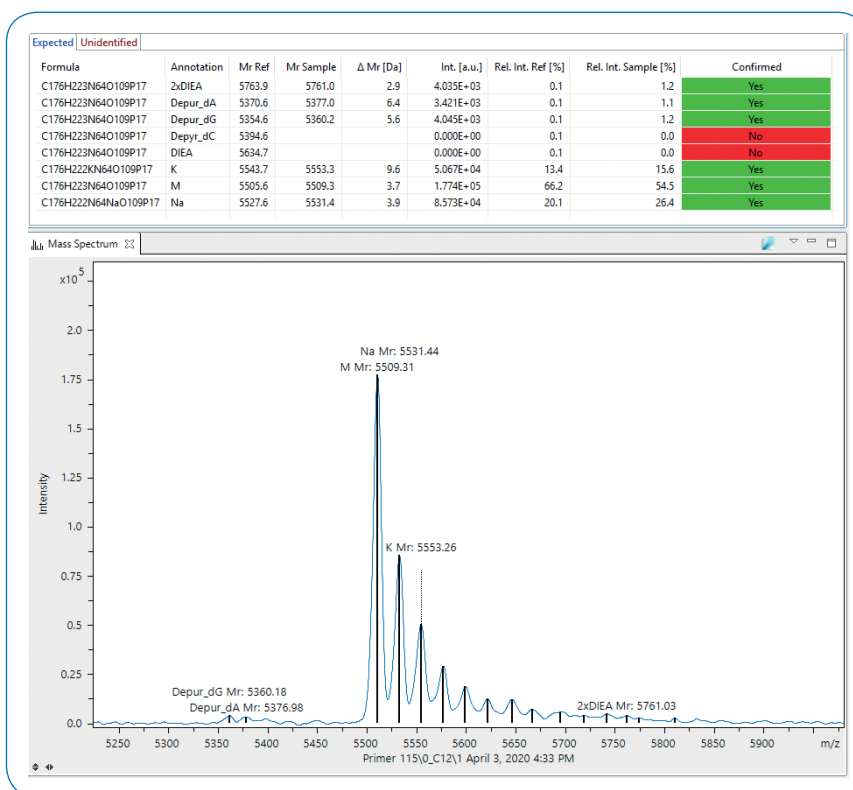


Figure 2: Result table with detailed description of the reference and the sample spectrum profiles and annotated mass spectrum of primer pET 3' at position C12. The peaks detected in the spectrum are the protonated form (54.5 %), the sodium and the potassium adduct in agreement with the expected reference profile.

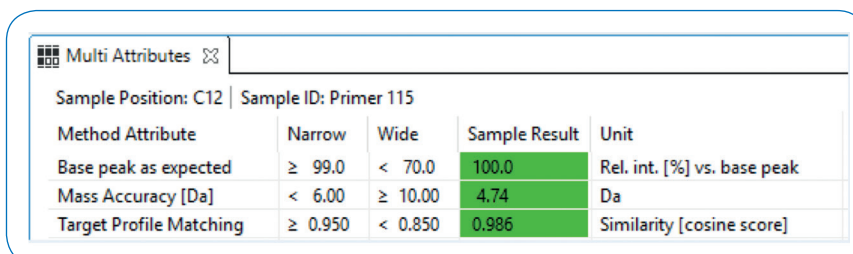


Figure 3: Summary of the quality attribute definitions used for oligo QC, including the actual results for position C12. All determined attributes are well agreed with the expectations and therefore marked in green.

name, spot ID and the molecular formula or molecular weight of the analyte oligonucleotide. Expected relative abundances, e.g., of sodium and potassium adducts and nucleobase-losses were defined as target profile (Figure 2, top: Rel. Int. Reference). An oligonucleotide has passed the quality control test when the mass spectrum matched the expected quality attributes. When a single target mass is of interest as in peptide or oligo QC, the expected mass should be the base peak of the spectrum within a certain mass error. In case of oligo nucleotides, intense adduct formation of sodium and potassium cannot be avoided in straight-forward MALDI preparation protocols. Therefore, salt adducts should be included in the profile for automated quality control. We used three out of six possible quality attributes in Biopharma Compass

2021 for the primer analysis. The 1st attribute was the Base peak as expected [%], which was set to 99 % and was reached by all primers, marked by the green color code. The 2nd attribute was mass accuracy, which was set between 6 Da and 10 Da (Figure 3). The majority of the spectra analyzed showed a mass accuracy of better than 6 Da and are marked in green. The similarity between expected peak profile and the peaks in the sample spectra was the 3rd attribute assessed by the cosine score (Figure 4). This attribute can be used to define a maximum amount of adduct formation accepted in oligo nucleotide analysis. It was translated in a color scheme: values higher than 0.9 (high similarity, marked in green), values lower than 0.6 (low similarity, red) with most samples marked in green. Medium similarity values were

marked in yellow, recommending further investigation of the result.

For each position, annotated spectra and detailed matching information (Figure 2) are provided in Biopharma Compass 2021. The expected peak profile and the measured profile are listed to inspect any differences and the spectrum is displayed to detect further, unexpected problems such as side products of the synthesis or other contaminations. Salt adducts were typically observed (Figure 2), in agreement with the synthetic primer's target sequence.

To fully interpret the color-coded quality attributes, a legend is provided for each sample, which summarizes their definitions and the obtained attribute values from the sample results (Figure 3).

Multi Target Screening MALDI Result Oligo QC Test_1.1



Primer 115

Row	Result	Position	Base Peak Mr [Da]	ΔBase Peak Mr [Da]	Sample Name	Target Profile Matching	Mass Accuracy [Da]	Base peak as expected
1	■ ■ ■ □ □ □	C12	5509.3	3.7	Primer 115	0.986	4.74	100.0

Expected

Row	Form	Annotation	Mr Ref	Mr Sample	Δ Mr [ppm]	Δ Mr [Da]	Int [a.u.]	Rel. Int. Ref [%]	Rel. Int. Sample [%]	Confirmed
1	C176H223N64 O109P17	2xDIEA	5763.9	5761.0	494.49	2.9	4.03E03	0.1	1.2	Yes
2	C176H223N64 O109P17	Depur_dA	5370.6	5377.0	1191.27	6.4	3.42E03	0.1	1.1	Yes
3	C176H223N64 O109P17	Depur_dG	5354.6	5360.2	1046.38	5.6	4.05E03	0.1	1.2	Yes
4	C176H223N64 O109P17	Depyr_dC	5394.6				0.00E00	0.1	0.0	No
5	C176H223N64 O109P17	DIEA	5634.7				0.00E00	0.1	0.0	No
6	C176H222KN6 4O109P17	K	5543.7	5553.3	1730.62	9.6	5.07E04	13.4	15.6	Yes
7	C176H223N64 O109P17	M	5505.6	5509.3	677.35	3.7	1.77E05	66.2	54.5	Yes
8	C176H222N64 NaO109P17	Na	5527.6	5531.4	702.11	3.9	8.57E04	20.1	26.4	Yes

Figure 4: Example PDF report of primer pET 3' at sample position C12.

As the described MALDI-based oligo-QC method does not involve any LC-separation, it is suitable for high throughput operation. Estimated times for the processing of 384 samples on a single 384 sample MALDI plate are 15 min for the robotic sample preparation – excluding preparation of the individual solutions loaded to the robot -, 30 min for spectra acquisition and processing, 5 min for data evaluation and report generation, amounting to approx. 50 min automatic processing time. Thus, approx. 10 sample plates á 384 samples can be verified using the described setup during an 8 h workday. Increased lab automation efforts (e.g., using 384 channel pipet heads and MTPs) can nearly quadruple that number of analyses per day.

Individual analysis reports were generated in PDF format (optionally as HTML or doc format) including result tables, mass spectra and method parameters. An example report is shown in Figure 4. Reports for an entire analysis set with 384 samples are also available.

The primers in the mass range <10 kDa can be reliably analysed by MALDI; no further analysis is typically required. For higher molecular weight (> 30-100 bp), however, the MALDI QC success rate decreases and a 2nd tier analysis using LC-ESI on QTOF-MS (e.g., Bruker compact or impact II) is helpful [3]. In such a case a 1st tier LC-free MALDI and 2nd tier LC-ESI analysis are combined, the fast MALDI QC can get the majority of the analyses completed and the slower LC-ESI QC can focus on the larger and the failed/more difficult oligonucleotide samples. BioPharma Compass supports the multi attributes analysis of LC-ESI QTOF data similarly to the MALDI QC described in this note, providing high throughput and consolidating the results in a single tool [4].

Conclusion

- A quality control analysis method is described, which includes the automated robot preparation of oligonucleotide samples such as primers, a fully automated mass spectrometry workflow including acquisition and the dedicated QC analysis of the MALDI-TOF mass spectra. It provides result visualization in the Biopharma Compass Software to allow users to get a quick overview as well as detailed information about the oligonucleotide samples.
- The described protocol provides for the analysis of up to 10 x 384 primer samples/8 hrs. Increased automation efforts may yield a much higher throughput using a single autoflex maX MALDI-TOF. In addition, the same protocol described here can also be used manually or in a semi-automatic manner for lower throughput requirements.



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