High-throughput mass spectrometry-based enzyme screening enabled by streptavidin magnetic beads

Samantha Fossa, B.Sc., Léa Chuzel, Ph.D., Michael Sproviero, Ph.D., Paul Caffrey, Ph.D.

INTRODUCTION

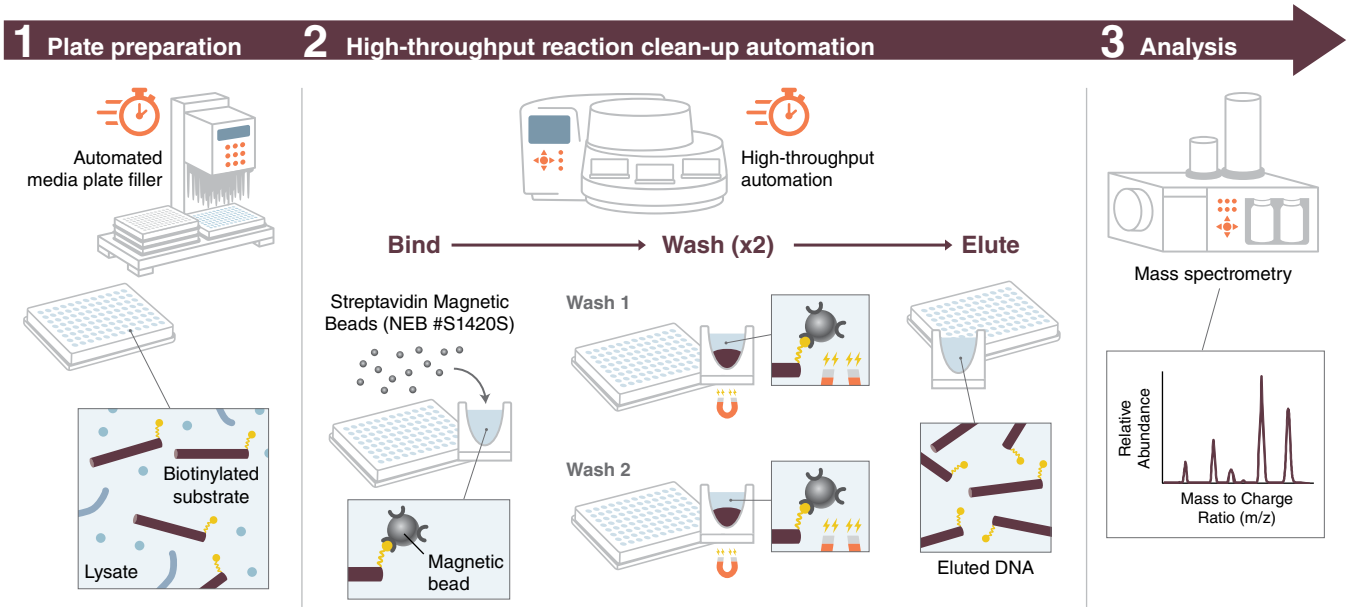
Despite widely available (meta)genomic sequencing data and an increasing number of gene prediction tools, many genes still have unknown functions or only broadly assigned activities (1–3). Historically, high-throughput functional screening has been an effective method for identifying new enzymes and assigning gene functions (4,5). This approach relies on constructing libraries containing fragments of genomic or environmental DNA into a vector backbone. We have recently used such a system in combination with high throughput capillary electrophoresis (CE) to discover novel, unannotated archaeal DNA modifying enzymes (6).

While CE is a powerful tool for enzyme discovery, it has several limitations. Firstly, it relies on a fluorescent moiety to be attached to the oligonucleotide for detection. Secondly, the enzymatic activity being investigated must result in a shift in migration time between the substrate and the product, preventing the detection of subtle modifications, such as alkylation. Additionally, if activity is indicated by a shift in signal, our understanding of the product's identity is often limited. It can be difficult to fully understand what the shift means other than a different size or charge. Finally, the number of fluorescent signals that can be multiplexed in a single assay limit the throughput to a handful of modifications.

MATERIALS

- Streptavidin Magnetic Beads (NEB #S1420S)
- KingFisher® Flex, automated purification instrument from ThermoFisher with 96 PCR head for KingFisher (#24074410)
- Agilent® 6545XT AdvanceBio® LC/Q-TOF
- Agilent UHPLC Guard AdvanceBio Oligo 2.1 x 5mm 2.7 Micron (#821725-921)
- Biotinylated oligonucleotide sequence(s) at 15 µM concentration in lysate

FIGURE 1: **Overview of the high-throughput screening methodology**



Clarified, heat-killed cell lysates were generated directly in a 96-well plate, followed by addition of biotinylated DNA substrate and incubation for 2 h at 55°C. Streptavidin Magnetic Beads were dispensed to the 96-well plate to clean up the reactions. The plate was placed in the magnetic particle processor (KingFisher Flex), which performs the binding, wash, and elution steps in approximately 1 h, yielding up to 96 purified samples ready for ESI-MS analysis.

In contrast, mass spectrometry (MS) allows for the detection of any modification that results in a mass difference and can be easily multiplexed to detect several modifications at once. A major drawback for MS-based detection is labor-intensive sample cleanup required to prepare samples in a MS- compatible buffer without interfering proteins, background DNA, and various other cellular material that can bias results, suppress signal, or interfere with instrument function.

NEB offers super-paramagnetic Streptavidin Magnetic Beads (NEB #S1420S), for highly specific binding of biotinylated biomolecules (i.e., nucleic acids and proteins). These beads are provided DNase/RNase free, ensuring compatibility is more concise with nucleic acid workflows. Since these beads can be easily dispensed to 96-well plates, they are particularly amenable to high-throughput workflow processes. We aimed to leverage these beads for sample preparation upstream of MS in an automated workflow using a magnetic particle processor (MPP), (Figure 1, page 1). The MPP automates purification of biotinylated DNA substrates from background nucleic acids, proteins, and cell debris by transferring the magnetic beads between plates containing purification buffers. Initially, lysate was incubated with biotinylated substrate, followed by specific pull-down of the biotinylated substrate with Streptavidin Magnetic Beads. Following binding, the MPP transferred the beads to plates containing wash buffer to remove contaminants. Finally, specific elution of the substrate into a plate containing elution buffer (wash buffer heated to 70°C) resulted in material immediately ready for MS analysis. We screened a hot spring metagenomic library for Uracil-DNA Glycosylase (UDG), a DNA repair enzyme that catalyzes the release of free uracil from uracil-containing DNA, in the presence of a normally inhibitory concentration of free uracil. This workflow resulted in the substrate and/or product oligonucleotide being in an aqueous, salt-free solution that allows for sensitive and reliable MS detection. Importantly, we demonstrate how users can screen hundreds of samples in a single day with minimal hands-on time. We also demonstrate normalized and maximized sample recovery from lysates, with only a single dropout sample observed from 2688 total samples screened.

PROTOCOL

Synthesis and preparation of the oligonucleotide substrate

The oligonucleotide used in this study was synthesized on a K&A H8 DNA synthesizer (SierraBio). All reagents were purchased from Glen Research. The oligonucleotide was synthesized trityl-on with standard protecting groups. The 3´ biotin was on the solid support from Glen Research. Following deprotection in 1 ml 1:1 ammonium hydroxide and methylamine at 65°C the samples were purified on GlenPak cartridges. On column detritylation was conducted before final elution of the oligos. Electrospray ionization mass spectrometry (ESI-MS) was used to verify the identity of the component oligonucleotide.

OLIGONUCLEOTIDE SEQUENCE	MOLECULAR WEIGHT
5´- TTC CAT GAG GGC TAG AAT TAC CTA**U**CCG GCC TCC TC /**3BioTEG**/ -3´	11502.89 Da

Modifications highlighted in bold are deoxyuridine (U) and 3´ biotin.

The oligonucleotide was prepared as a 2X substrate master mix as 30 µM oligo, 20 mM Uracil, 100 mM Tris-HCl, 10 mM $MgCl_2$, 2 mM ATP, pH: 7.5.

Lysate preparation and screening reactions

1. Clones were grown in 1 ml LB medium containing 12.5 µg/ml chloramphenicol and 1X auto-inducing solution (500X auto-inducing solution is 10% arabinose, 10% glucose in water) at 37°C overnight.
2. The cells were pelleted at 2500 rpm for 5 min.
3. The supernatant was removed, and cell pellets resuspended in 100 µl of buffer containing 20 mM Tris-HCl pH 7.5 and 50 mM NaCl.
4. The resuspended cells were sonicated in a Q700 Sonicator by Qsonica® with Microplate Horn System at 50% amplitude for 10 min.
5. The lysates were clarified by centrifugation at 2500 rpm for 5 min. The supernatant was transferred to a new plate.
6. The lysates were heat-treated at 55°C for 1 h to deactivate host nucleases.
7. Screening reactions were prepared by mixing a volume of 10 µl lysate and 10 µl of 2X substrate master mix containing 30 µM biotinylated oligonucleotide and cofactors.
8. Reactions were incubated at 55°C for 2 h.

Streptavidin magnetic beads preparation

1. A volume of 1.5 ml of Streptavidin Magnetic Beads was transferred into a microcentrifuge tube.
2. The tube was placed on a magnetic rack and the supernatant was removed once clear.
3. The tube was removed from the magnetic rack, and 1 ml of high-salt buffer (20 mM Tris-HCl, 1 mM EDTA, 0.5 M NaCl, pH 7.5) was added to resuspend the beads. The beads were vortexed, and the tube was placed back on the magnetic rack and supernatant was removed once clear.
4. Step 3 was repeated two additional times for a total of 3 washes.
5. The beads were resuspended in 1 ml of high-salt buffer and transferred to a 5 ml or 15 ml tube. An additional 3 ml of high-salt buffer was added for a final volume of 4 ml of prepared beads. Beads were kept in suspension via end-over-end mixing until ready to dispense.

KingFisher Flex-aided sample clean-up

1. Using a VIAFLO® 96 (alternative: 200 µl multichannel pipette) each well of a fully skirted 96-well PCR plate was filled with 100 µl of high-salt buffer. The plate was labeled '**wash 1**'.
2. Using a VIAFLO 96 (alternative: 200 µl multichannel pipette) each well of a fully skirted 96-well PCR plate was filled with 100 µl of low-salt buffer (20 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, pH 8). The plate was labeled '**wash 2**'.
3. Using a VIAFLO 96 (alternative: 200 µl multichannel pipette) each well of a fully skirted 96-well PCR plate was filled with 40 µl of low-salt buffer. The plate was labeled '**elution**'.
4. Using a VIAFLO 96 (alternative: 200 µl multichannel pipette) 20 µl of reaction was mixed with 20 µl of high-salt buffer and 40 µl of prepared Streptavidin Magnetic Beads in each well of a fully skirted 96-well PCR plate. The plate was labeled '**sample**'.
5. One 96 PCR tip comb for KingFisher magnets was placed in a fully skirted 96-well PCR plate. The plate was labeled '**tip comb**'.
6. A new method was created on the KingFisher Flex instrument which included the 5 plates outlined above ('**tip comb**', '**sample**', '**wash 1**', '**wash 2**' and '**elution**').
7. In the instrument method, tips were set as '**PCR tip comb**' and 6 steps were created with parameters as outlined in Table 1.
8. The 96 PCR head on the KingFisher Flex was installed.
9. The method was started and the 5 x 96-well fully skirted PCR plates ('**wash 1**', '**wash 2**', '**elution**', '**sample**' and '**tip comb**') were placed in appropriate locations.
10. Once the method was completed (~ 1 h), the plates were collected from the instrument. The plate '**elution**' contained the cleaned samples.
11. To ensure no beads remained in the samples, the plate '**elution**' was placed on a 96-well magnet plate and the supernatants were transferred to a clean plate.

TABLE 1: **Parameters of the KingFisher Flex method**

STEP #	PLATE	PARAMETERS			
Step 1: Pick-up	**Tip comb**	"**Tip comb**": 96 PCR plate			
		BEGINNING OF THE STEP	**MIXING**	**HEATING**	**END OF STEP**
Step 2: Bind	**Sample**	No pre-collect no release beads	• Slow speed for 55 seconds • Medium speed for 5 seconds • Loop count 30	No	Collect beads Count 5 for 20 seconds
Step 3: Wash 1	**Wash 1**	Release beads For 00:00:05 medium speed	• Slow speed for 5 minutes • Medium speed for 5 seconds • Loop count 1	No	Collect beads Count 5 for 10 seconds
Step 4: Wash 2	**Wash 2**	Release beads For 00:00:05 medium speed	• Slow speed for 2 minutes • Medium speed for 5 seconds • Loop count 1	No	Collect beads Count 5 for 10 seconds
Step 5: Elution	**Elution**	No release beads For 00:00:05 medium speed	• Slow speed for 15 minutes	70°C	Collect beads Count 5 for 10 seconds
Step 6: Leave	**Tip comb**	"tip comb" in a 96 PCR plate			

Data acquisition on QToF

1. A worklist was prepared to inject 20 µl of sample with substrate masses indicated.
2. A fast desalting method was run as described in Table 2:

TABLE 2: **Mobile phase gradient**

TIME (MINUTES)	MOBILE PHASE A (60 mM HFIP, 7.7 mM DIPEA IN WATER) (%)	MOBILE PHASE B (METHANOL) (%)	FLOW (ml/min)
0.00	80	20	0.6
0.13	80	20	0.6
0.34	40	60	0.6
0.35	10	90	0.6
0.45	10	90	0.6
0.46	80	20	0.6
0.74	80	20	0.6

3. The data were processed using BioConfirm software with appropriate deconvolution ranges for target oligo(s).
4. Processed data were opened in BioMS Reviewer for high-throughput visualization. Thresholds for detection can be set and multiple targets can be programmed for easy evaluation of an otherwise unwieldy dataset.

TABLE 3: **Mass spectrometer settings**

SOURCE CONDITIONS	
Gas Temp	300°C
Drying Gas	12 L/min
Nebulizer	60 psi
Sheath Gas Temp	300°C
Sheath Gas Flow	12 L/min
Vcap	3500 V
Nozzle Voltage	2000 V
Fragmentor	200 V
Skimmer	65 V
Oct 1 RF Vpp	750 V

RESULTS

Sample preparation with magnetic beads permits robust, high-throughput plate-based screening

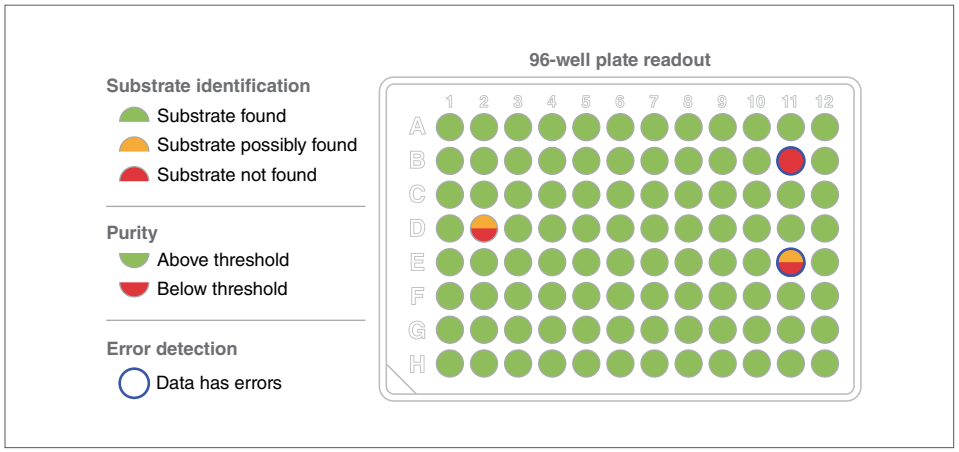
We screened 28 x 96-well plates, corresponding to 2688 distinct samples that each contained the lysate of a unique metagenomic clone. Only one sample dropout where no MS signal corresponding to substrate or product was observed (dropout rate of 0.04%) indicating robust binding/washing/elution conditions. Furthermore, the ratio of beads to substrate was designed to induce saturation of the beads and normalize the amount of sample in each well. This additional benefit eliminated the need for quantification of samples across the wells and enabled a consistent injection volume.

MS data were conveniently surveyed for the presence and relative abundance of the substrate using BioMS reviewer. A representative overview of a 96-well plate readout can be seen in Figure 2. The target mass was assigned to the unmodified substrate mass (11502.89 Da), samples were flagged (the bottom half of the circle turns red) when less than 30% relative abundance of the substrate remained (this value can be tailored to the specific application using a simple slider bar in the BioMS reviewer software) or when no target mass is observed (the entire circle will turn red). Most of the wells in Figure 2 remained fully green. The three wells with a red lower color (Figure 2, wells B11, D2 and E11) were further investigated using BioConfirm.

Detection of UDG activity with BioConfirm

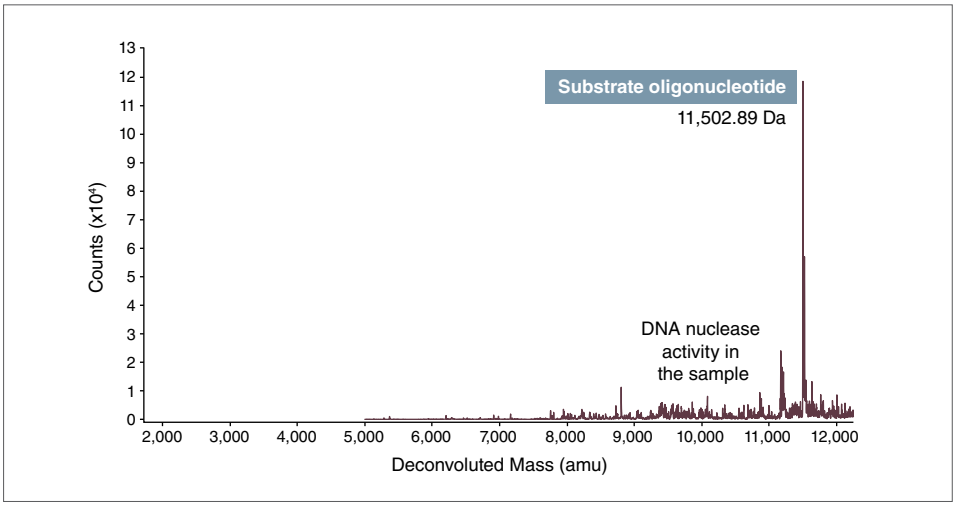
The three samples (B11, D2 and E11) where substrate had been flagged as altered using BioMS Reviewer were further investigated with BioConfirm to determine the specific mass differences detected. We observed that the sample in B11 did show the substrate mass (Figure 3) but at a lower amount than expected and far below the 30% threshold limit that was set for the BioMS Reviewer analysis. Based on the presence of other shorter oligos we surmised that nuclease activity (that survived the heat treatment step) was present in this well (Figure 3).

FIGURE 2: **Representative overview of a 96-well plate readout using BioMS reviewer**



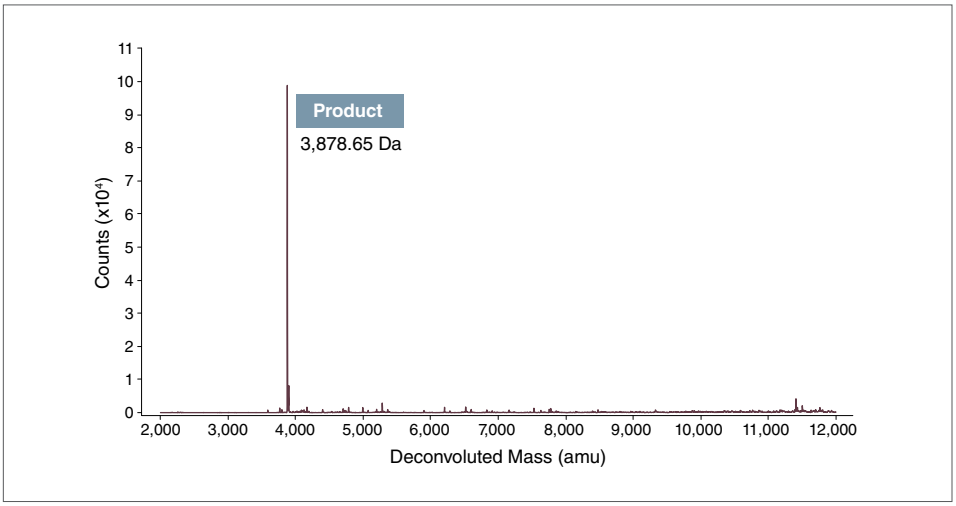
The upper half of each well indicates presence/absence of target mass. The lower half of each well represents the relative abundance of the target mass; the value is set by the user.

FIGURE 3: **Deconvoluted mass spectrum of sample B11**



A mass corresponding to the substrate oligonucleotide is observed at 11502.89 Da. An array of smaller masses indicates the potential presence of DNA nucleases in the sample.

FIGURE 4: **Deconvoluted mass spectrum of sample D2**



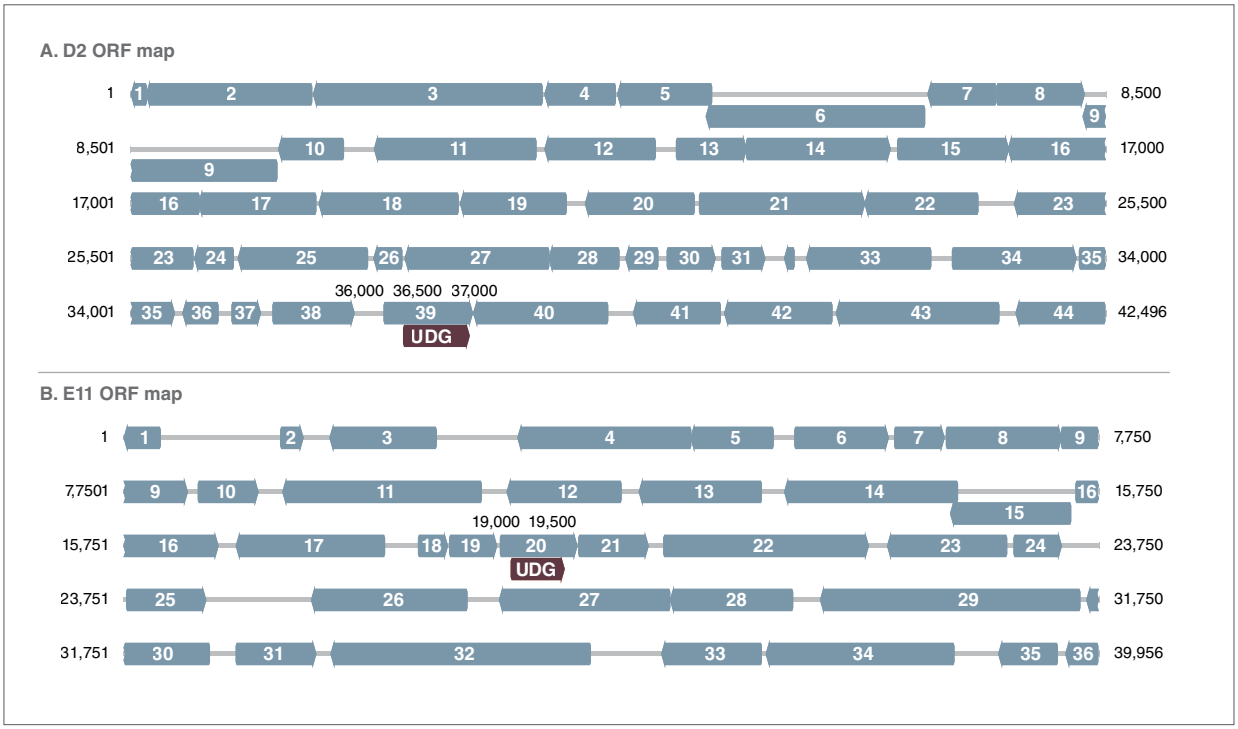
A mass at 3878.65 Da corresponding to the product was observed in sample D2. The product corresponds to the cleavage of the oligonucleotide substrate at the Uracil site upon UDG activity.

However, for both samples D2 (Figure 4) and E11 we observed a pattern indicating the formation of an apyrimidinic (AP site) at the dU location and subsequent strand cleavage. Taken together, these results indicated the potential presence of a UDG resistant to the free uracil inhibitor we added (see Protocol). We therefore identified the clones in these wells as potential candidates for UDG activity. Both clones were chosen for sequencing and bioinformatic analysis.

Bioinformatic confirmation of potential UDG genes

With the observation of masses corresponding to UDG products, we investigated whether any genes in clones D2 and E11 could be identified as encoding a UDG. To that end, fosmids from clones D2 and E11 were sequenced and open reading frames (ORFs) predicted. ORF annotations confirmed two different UDG candidates, one in each clone (Figure 5).

FIGURE 5: **ORF maps of clones D2 and E11**



Sequences of clones D2 and E11 were used to predict ORFs, represented in blue/grey. ORF39 and ORF20 on clone D2 and E11 respectively, are annotated as UDG (UDG domain found).

CONCLUSION

Maximizing throughput in enzymatic discovery is essential to find novel and elusive activities of interest. Streptavidin Magnetic Beads are particularly amenable to high-throughput screening workflows as they enable enrichment of biotinylated molecules and are easily manipulated in parallel with the use of magnetic particle processors and/or magnetic racks that can hold 96-well plates. Furthermore, elution is possible in MS compatible buffers that minimize downstream sample processing.

The ability to detect the activities of nucleic acid modifying enzymes has traditionally required modification of the substrate with a fluorophore or radiolabel and a migration change as a result of the enzymatic activity. MS-based detection allows for the direct detection of enzymatic activity identified through a mass difference of the substrate. While LC-MS workflows traditionally require a labor-intensive workup before injection we demonstrate here a robust, high-throughput MS-based methodology enabled by Streptavidin Magnetic Beads and an automated KingFisher purification system. Streptavidin Magnetic Beads showed consistent high recovery of biotinylated DNA from complex lysates, generating normalized samples that are immediately ready for LC-MS analysis. This methodology was used to detect the activity of novel UDGs. We envision these beads can be used with a variety of biotinylated substrates across a wide range of workflows to elucidate enzymatic activities that induce a mass change.

References

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