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DNA sequence recognition by DNA-primase using high-throughput primase profiling (HTPP)

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March 26, 2019

Dear Dr. DSouza,

We are submitting a revised version of our manuscript JoVE59737, entitled "**DNA sequence recognition by DNA-primase using high-throughput primase profiling (HTPP)**".

In response to the reviewer comments, we further clarified the text and added new explanatory notes as elaborated in the response letter. As this paper provides an overview of the methodology (FHTPP) and didn't aim to present new data, we think that the modified version provide an extended view on the method and do not repeat in any way the published data on the recognition by T7 primase.

Overall, we believe our revised manuscript is much stronger, addresses the reviewers' comments, and describes the implications of primase binding and functional activity. Thus, we hope you will consider our revised manuscript for publication in *JOVE*.

Sincerely,

Barak Akabayov

Barak Akabayov

TITLE:

DNA Sequence Recognition by DNA Primase Using High-Throughput Primase Profiling

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KEYWORDS:

high throughput primase profiling, HTPP, protein binding microarray, PBM, DNA primase, DNA replication, protein-DNA interaction, DNA-binding, RNA primers, molecular biology

SUMMARY:

Protein binding microarray (PBM) experiments combined with biochemical assays link the binding and catalytic properties of DNA primase, an enzyme that synthesizes RNA primers on template DNA. This method, designated as high-throughput primase profiling (HTPP), can be used to reveal DNA-binding patterns of a variety of enzymes.

ABSTRACT:

DNA primase synthesizes short RNA primers that initiate DNA synthesis of Okazaki fragments on the lagging strand by DNA polymerase during DNA replication. The binding of prokaryotic DnaG-like primases to DNA occurs at a specific trinucleotide recognition sequence. It is a pivotal step in the formation of Okazaki fragments. Conventional biochemical tools that are used to determine the DNA recognition sequence of DNA primase provide only limited information. Using a high-throughput microarray-based binding assay and consecutive biochemical analyses, it has been shown that 1) the specific binding context (flanking sequences of the recognition site) influences the binding strength of the DNA primase to its template DNA, and 2) stronger binding of primase to the DNA yields longer RNA primers, indicating higher processivity of the enzyme. This method combines PBM and primase activity assay and is designated as high-throughput primase profiling (HTPP), and it allows characterization of specific sequence recognition by DNA primase in unprecedented time and scalability.

INTRODUCTION:

HTPP makes use of DNA binding microarray technology combined with biochemical analysis (**Figure 1**) to statistically identify specific features of DNA templates that affect the enzymatic activity of DNA primase. Therefore, HTPP provides a technological platform that facilitates a knowledge leap in the field. The classical tools used to determine primase recognition sites do not have the ability to yield massive amount of data, whereas HTPP does.

PBM is a technique routinely used to determine the binding preferences of transcription factors to DNA[1,2]; however, it is not suitable for detection of weak/transient binding of proteins to DNA. Unlike the universal PBM that provides information about average protein binding specificity to all possible sequences consisting of eight base pairs, HTPP is based on the library of single-stranded DNA templates comprising unique sequence elements. Such DNA sequence elements involve tens of thousands short (few tens of bp) genomic sequences, as well as computationally designed DNA sequences enriched in certain DNA repetitive sequence elements present in the genome, which possess different average GC content. Such a high-throughput approach allows determination of, in a systematic, quantitative, and hypothesis-driven way, the sequence-related properties that are important for primase binding and its enzymatic activity[3]. In particular, the important link between primase-DNA binding preferences, (modulated by DNA sequences flanking specific tri-nucleotide binding sites) and primase processivity has been identified for this enzymatic system[4].

The new technology was applied to revisit our understanding of primase recognition sites even for the T7 DNA primase that has been extensively studied[5]. Specifically, re-examination of classical concepts, such as DNA recognition sites of T7 DNA primase (which were determined almost four decades ago [6]) using protein-DNA binding microarray (PBM) has led to unprecedented insight into features related to the flanking sequence of these recognition sites[3]. It was expected that the sequences flanking tri-nucleotide recognition site of T7 DNA primase (5'-GTC-3') will be random. Instead, we found that TG-rich flanking sequences increase the chances of T7 DNA primase to synthesize longer RNA primers indicating an increase in processivity.

Other methods that can be used to study DNA-binding properties of proteins in vitro include the electrophoretic mobility shift assay (EMSA)[7], DNase I footprinting[8], surface-plasmon resonance (SPR)[9], and Southwestern blotting[10]. These are, however, low-throughput methods only applicable to investigating a small number of DNA sequences. In addition, the precision and sensitivity of some of these techniques (e.g., EMSA) is low. On the other hand, in vitro selection[11] is a technique that, similarly to PBM, can be used for the identification of numerous binding sequences. However, low affinity sequences are usually excluded in most applications of in vitro selection; therefore, this approach is not suitable for obtaining comparative binding data for all available sequences. The universal PBM[1,2] is mainly used to characterize the binding specificities of transcription factors from prokaryotes and eukaryotes as well as specific factors (e.g., presence of certain ligands, cofactors, etc.) that may affect this interaction[12].

HTPP expands the PBM application to DNA processing enzymes by combining unprecedented high-throughput statistical power with high precision to provide information on binding sequence context. Such data has not yet been obtained for primases and related enzymes (that have weak/transient binding to DNA) due to aforementioned technical limitations of other available techniques.

PROTOCOL:

1. Design of microarray

NOTE: DNA probes represent custom 36-nucleotide sequences, consisting of the recognition site for T7 DNA primase (GTC) located between two variable flanking regions, followed by a constant 24-nucleotide sequence tethered to a glass slide[3]. We used a 4 x 180,000 microarray format, which enabled spotting of each DNA sequence in six replicates, randomly distributed on the slide.

1.1. Design of DNA library for primases with known recognition sequences

1.1.1. Ensure that each sequence is composed of 60 nucleotides. Keep the first 24 nucleotides constant (to be attached to the glass slide). Variable regions should have the following general form: $(N)_{16}GTC(N)_{17}$; where N represents any desired nucleotide.

1.1.2. Design the flanking region to address a specific scientific question (an example of the design is presented in the following text). The flanking regions may be designed to contain specific features such as the repeat elements or specific symmetry.

NOTE: We have created eight categories of different flanking sequences composed of two or three specific nucleotides: T and G (group 1); T and C (group 2); C and A (group 3); A and G (group 4); G, C and T (group 5); C, T and A (group 6); G, A and T (group 7); G, A and C (group 8). 2,000 different sequences were designed for each group. Each group had subgroups of sequences with different types and different numbers of sequence repeats.

1.1.3 Include a set of negative control sequences (lacking the specific binding site)[4]. The presence of such sequences allows to validate the occurrence of specific binding in the experiment.

1.2 Design of DNA library for primases with unknown recognition sequences

1.2.1. If the recognition sequence is unknown, create the DNA library by selecting the sequences (with or without specific features mentioned previously) from the genome of desired organism (bacteria, fungi, etc.).

1.3 Design of microarray slide

1.3.1. Purchase custom slides (e.g., 4x180K, AMADID #78366) from a commercial supplier (for more details see **Table of Materials**). Order each sequence in six replicates, where each replicate should be attached to a randomly selected spot on the slide.

2. Primase DNA binding experiment

NOTE: The day before (or at least 2 h before) the PBM, prepare the blocking solution [2% w/v skim milk in phosphate buffer saline (PBS)] and stir it on a magnetic stirrer. Prior to use, filter the solution with a 0.45 µM filter. To detect the primase binding to the DNA strands, several steps should be performed in the following order.

2.1 Blocking procedure

2.1.1. Pre-wet the microarray slide in a Coplin jar with 0.01% v/v Triton X-100 in PBS (5 min at 125 rpm on a lab rotator).

2.1.2. Briefly wash the gasket slide (also referred to as coverslip) with water and ethanol and dry using compressed air.

2.1.3. Assemble bottom part of steel hybridization chamber (PBM chamber) with the gasket slide facing up (commercial label facing up). For more details regarding the assembly, refer to the **Table of Materials**.

2.1.4. Pipet blocking solution (2% w/v skim milk in PBS) into each chamber.

2.1.5. Remove the microarray slide from the Coplin jar, then dry the non-DNA side (the DNA should be on the same side as the company label) and edges using a fine wipe. Slowly place the microarray on the gasket slide, avoiding bubbles (company label facing down). Immediately assemble and tighten steel hybridization chamber apparatus. Incubate for 1 h at room temperature (RT).

2.1.7. Fill one staining dish (the “PBS” dish) with 800 mL of fresh PBS. Fill a second staining dish (the “WASH” dish) with 800 mL of freshly prepared 0.5% v/v Tween-20 in PBS.

2.1.8. Unscrew the PBM chamber and remove the microarray slide-coverslip “sandwich”, taking care not to break the seal. Disassemble it underwater in the WASH dish by placing forceps between the slide and coverslip.

2.1.9. Shake the microarray slide underwater and quickly transfer to a Coplin jar.

2.1.10. Wash once with 0.1% v/v Tween-20 in PBS (5 min at 125 rpm on a lab rotator).

2.1.11. Wash once with 0.01% v/v Triton X-100 in PBS (2 min at 125 rpm on a lab rotator).

2.1.12 Quickly transfer the slide to a Coplin jar containing PBS.

2.2 Protein binding

2.2.1. Assemble the **PBM chamber** as previously described (steps 2.1.2–2.1.3).

2.2.2. Pipette protein binding mixture containing 5 µM T7 DNA primase, 6.5 mM $MgCl_2$, 30 mM K-glutamate, 6 mM dithiothreitol (DTT), 65 µM ribonucleoside triphosphate (rNTP), 2% w/v skim milk, 100 ng/µL bovine serum albumin (BSA), 50 ng/µL salmon testes DNA, and 0.02% v/v Triton X-100 (TX-100) into each chamber of the gasket slide (without touching the rubber sides and without introducing bubbles).

2.2.3. Rinse the microarray slide briefly by dipping it in the PBS dish, which removes excess detergent from the surface of the slides. Wipe the non-DNA surfaces of the slide with a fine wipe.

2.2.4. Slowly lower the microarray slide (facing down) onto gasket slide, being careful to prevent leakage from one chamber to another. Immediately assemble and tighten PBM chamber apparatus without introducing bubbles. If bubbles do form, they can be removed by gently tapping the steel chamber against a hard surface.

2.2.5. Incubate for 30 min at room temperature (RT).

2.3 Fluorescent antibody attachment

2.3.1. Unscrew the PBM chamber and remove the microarray slide-coverslip "sandwich", taking care not to break the seal. Disassemble underwater in the WASH dish using forceps. Shake the slide underwater and quickly transfer to a Coplin jar containing PBS.

2.3.2. Assemble the PBM chamber with gasket slide as previously described (steps 2.1.2–2.1.3).

2.3.3. Add Alexa 488-conjugated anti-his antibody (10 ng/µL in binding buffer) to the gasket slide.

2.3.4. Rinse the slide briefly by dipping it in PBS dish as before, then remove the slide from PBS slowly, wipe the non-DNA surface and place it facing down onto gasket slide.

2.3.5. Incubate 30 min at RT in the dark (fluorescence probe is light- sensitive) to reduce photo-bleaching.

2.3.6. Disassemble the PBM chamber and coverslip as before, removing the coverslip underwater in the WASH dish.

2.3.7. Rinse the slides briefly by dipping them in the PBS dish. Dry the slides with compressed

air. Store in the dark in a slide box until ready to scan.

2.4. Scanning the microarray slide

2.4.1. Scan the chip by using microarray scanner (refer to **Table of Materials**) with excitation of 495 nm and emission of 519 nm and collect the median fluorescence intensity.

3. Microarray data analysis

NOTE: All data processing was performed using custom written scripts in MATLAB.

3.1. Perform the initial PBM data processing using Wilcoxon rank sum test p-value as described previously[4].

3.2. Use the median value of the binding intensity for each DNA sequence for further analysis.

3.3. Next, using the one-way ANOVA p-values, compare statistical significance of the observed differences in primase-DNA binding intensities obtained for different groups of DNA probes, as explained above in the DNA library design section[3].

4. Template-directed RNA synthesis catalyzed by T7 DNA primase

4.1. Preparation of denaturing polyacrylamide gel

4.1.1. To prepare 100 mL of gel mixture, combine 62.5 mL of 40% acrylamide-bisacrylamide (19:1), 42 g of urea, 1.1 g of Tris base (2-Amino-2-(hydroxymethyl)-1,3-propanediol), 0.55 g of boric acid, and 0.4 mL of 0.5 M ethylenediaminetetraacetic acid (EDTA) solution.

4.1.2. Divide the mixture into 14 mL aliquots (the amount needed for one gel of 16.5 cm x 26 cm x 0.3 cm) and keep them protected from light at 4 °C for up to 1 month.

4.1.3. To prepare one denaturing polyacrylamide gel, add 4 µL of TEMED and 40 µL of 10% w/v ammonium persulfate to 14 mL of the previously prepared mixture, cast it, and leave it to polymerize for at least 2 h at RT. The gel can be kept for one day at 4 °C.

4.2. Sample preparation

NOTE: Keep the reagents, reaction mixture, and samples on ice.

4.2.1. To prepare the reaction mixture required for ten reactions combine 11 µL of 5x activity buffer (200 mM Tris-HCl, pH = 7.5; 50 mM dithiothreitol, 250 mM potassium glutamate, 50 mM $MgCl_2$), 5.5 µL of 2.5 mM mixture of nucleotide tri-phosphate (NTPs), and 5.5 µL of radiolabeled ribonucleotide [e.g., ATP, (α-32P) 3000 Ci/mmol].

NOTE: All amounts have been increased by 10% due to possible pipetting errors. Radiolabeled ribonucleotides should be diluted according to the strength of radioactive signal (initial dilution is usually 1:10 or more).

4.2.2. Transfer 2 µL of the reaction mixture to ten PCR tubes.

4.2.3. Add 1 µL of 100 µM DNA template to the corresponding PCR tube and spin down.

4.2.4. Add 2 µL of 16 µM T7 DNA primase, spin down, mix gently, and spin down again.

4.2.5. Incubate the reaction at RT for 20 min.

4.2.6. Stop the reaction by adding 5 µL of quenching solution (95% formamide, 20 mM EDTA, 0.05% w/v xylene cyanol and bromophenol blue), mix, and spin down.

4.3. Separation of RNA products by electrophoresis through denaturing polyacrylamide gel and subsequent signal detection

4.3.1. Pre-run the gel for 1 h (10 mA, 600 V) in 1x TBE buffer (90 mM Tris-borate, 2 mM EDTA).

4.3.2. Load up to 2 µL of each sample and perform electrophoresis (20 mA, 1100 V) in 1x TBE buffer (90 mM Tris-borate, 2 mM EDTA).

4.3.3. Dry the gel for 2 h at 80 °C under a vacuum.

4.3.4. Expose the phosphor imaging plate to radioactive gel for a few hours to overnight, depending on the strength of radioactive signal.

4.3.5. Record the signal (photostimulated luminescence) by using phosphorimager (see **Table of Materials**).

REPRESENTATIVE RESULTS:

This technological advance for mapping the primase binding sites allows the obtaining of DNA binding properties that are difficult, if not impossible, to observe using classical tools. More importantly, HTPP enables the revisiting of the traditional understanding of primase binding sites. Specifically, HTPP reveals binding specificities in addition to known 5'-GTC-3' recognition sequences, which leads to changes in functional activities of T7 DNA primase. Namely, two groups of sequences were identified: strong-binding DNA sequences that contained T/G in the flanks and weak-binding sequences that contained A/G in the flanks (all thymines in the strong-binding templates were replaced by adenines). No primase binding to DNA templates that were missing 5'-GTC-3' within their sequence was detected.

The primase DNA recognition sites that contained specific features, such as T/G-rich flanks,

increased primase-DNA binding up to 10-fold, and surprisingly also increased the length of newly formed RNA (up to threefold) (Figure 2 in previous publication[3]). Importantly, HTPP allowed us to observe and quantify the variability in primer length in relation to the sequence of the DNA template.

FIGURE LEGENDS:

Figure 1: Schematic representation of high-throughput primase profiling (HTPP). (**A**) The slide was incubated with the primase in activity buffer (40 mM Tris-HCl, pH 7.5; 10 mM $MgCl_2$, 50 mM K-glutamate, 10 mM DTT, 100 µM rNTPs). Next, Alexa 488-conjugated anti-his fluorescent antibody was introduced to label the protein. After the mild washing step, the slide was scanned using microarray scanner. Binding affinity was determined according to the median fluorescence signal and DNA sequences were divided into groups accordingly. (**B**) Biochemical assays were performed to correlate the DNA-binding results obtained from the microarray experiment, with the functional properties of T7 DNA primase.

Figure 2: Comparison of catalytic activity of T7 DNA primase on two groups of DNA templates (strong binding with T/G in the flanks and weak binding with A/G in the flanks) obtained from PBM. (**A**) RNA primer formation catalyzed by the T7 DNA primase. The reactions contained oligonucleotides with the primase recognition sequence (numbered lanes), ^{32}P-α-ATP, ATP, CTP, UTP, and GTP in the standard reaction mixture. One group of DNA oligonucleotides (dark blue) contained T/G in the flanks, whereas all thymines were replaced with adenines in the second group (light blue). After incubation, the radioactive RNA products were separated by electrophoresis through a 25% polyacrylamide gel containing 7M urea and visualized by autoradiography. (**B**) Relative length (number of ribonucleotides constituting each RNA primer is unknown) and amount of RNA primers synthesized by T7 DNA primase on two groups of DNA templates (panel A). The plots show that longer RNA primers are synthesized (increased processivity) on DNA templates that primase binds with higher affinity (that contain T/G in the flanks) compared to the templates that are bound with lower affinity (that contain A/G in the flanks). (**C**) Quantification of the amount of synthesized RNA primers on two groups of DNA templates. The results demonstrate a correlation between the DNA-binding affinity and the amount of synthesized RNA by the T7 DNA primase. AU (arbitrary unit) is the measure of intensity of radioactive signal which directly correlates with the amounts of synthesized RNA primers.

DISCUSSION:

The PBM method has been widely used to investigate binding properties of transcription factors and can also be applied to DNA processing enzymes, such as DNA primase, that bind to DNA with low affinity. However, certain modifications of experimental procedures are required. The microarray experiment involves several steps: design of the DNA library, preparation of the chip, binding of the protein target, fluorescent labeling, and scanning. Mild washing steps are critical, since the long washes with solutions containing detergents cause dissociation of the protein from the DNA template due to the weak/transient mode of binding. Other critical steps

include binding conditions (e.g., buffer composition, cofactors) and incubation times that need to be optimized for each specific enzyme.

The results obtained from microarray need to be validated in biochemical assays. Biochemical assays also provide insight into interesting features of DNA processing enzymes such as the correlation between DNA binding affinity and enzymatic activity/processivity. HTPP enabled us to observe the effect of DNA binding on functional properties of T7 DNA primase. For example, it has been observed that if primase exhibits higher binding affinity for the DNA template, it catalyzes the formation of longer RNA primers (increased processivity).

Overall, the method presented in this article is fast, reliable, and provides the opportunity to simultaneously test binding properties on tens of thousands of diverse DNA sequences in a single experiment in a hypothesis-driven way. Addition of other proteins or metal cofactors to the reaction mixture offers the possibility to investigate their effect on DNA binding properties of primases in a fast, high-throughput manner. On the other hand, the application of this method is limited by relatively high price of microarray slides and the safety precautions required when handling radioactive material for primase activity assays.

It is important to note that different primases require modifications of both microarray binding conditions and buffer conditions for activity assays. For example, primase of *Mycobacterium tuberculosis* requires substitution of magnesium with divalent manganese in the reaction buffer. Inappropriate metal cofactors or inappropriate reaction buffers may lead to poor primase activity or decreased DNA binding affinity.

As mentioned previously, primases bind to DNA templates with weak affinity; therefore, gentle washing steps are required during microarray binding experiment. Otherwise, they will be washed out from the microarray slide, leading to loss of the florescent signal upon addition of fluorescently labeled antibody.

To summarize, the DNA binding properties of many prokaryotic primases (including the trinucleotide DNA recognition sequence) are still poorly understood, mostly due to the technical limitations of currently available methods. HTPP represents a fast and efficient platform for the discovery of the DNA recognition sites or investigations of other template-related factors (i.e., overall nucleotide composition, GC content, presence of repetitive DNA sequence elements and their symmetry) that affect the binding affinity and activity of ssDNA binding enzymes. In addition, future applications may be directed towards the effects of different proteins or cofactors on DNA binding affinity, recognition patterns, or functional activity of primases and other DNA processing enzymes.

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DISCLOSURES:
The authors declare no conflict of interest.

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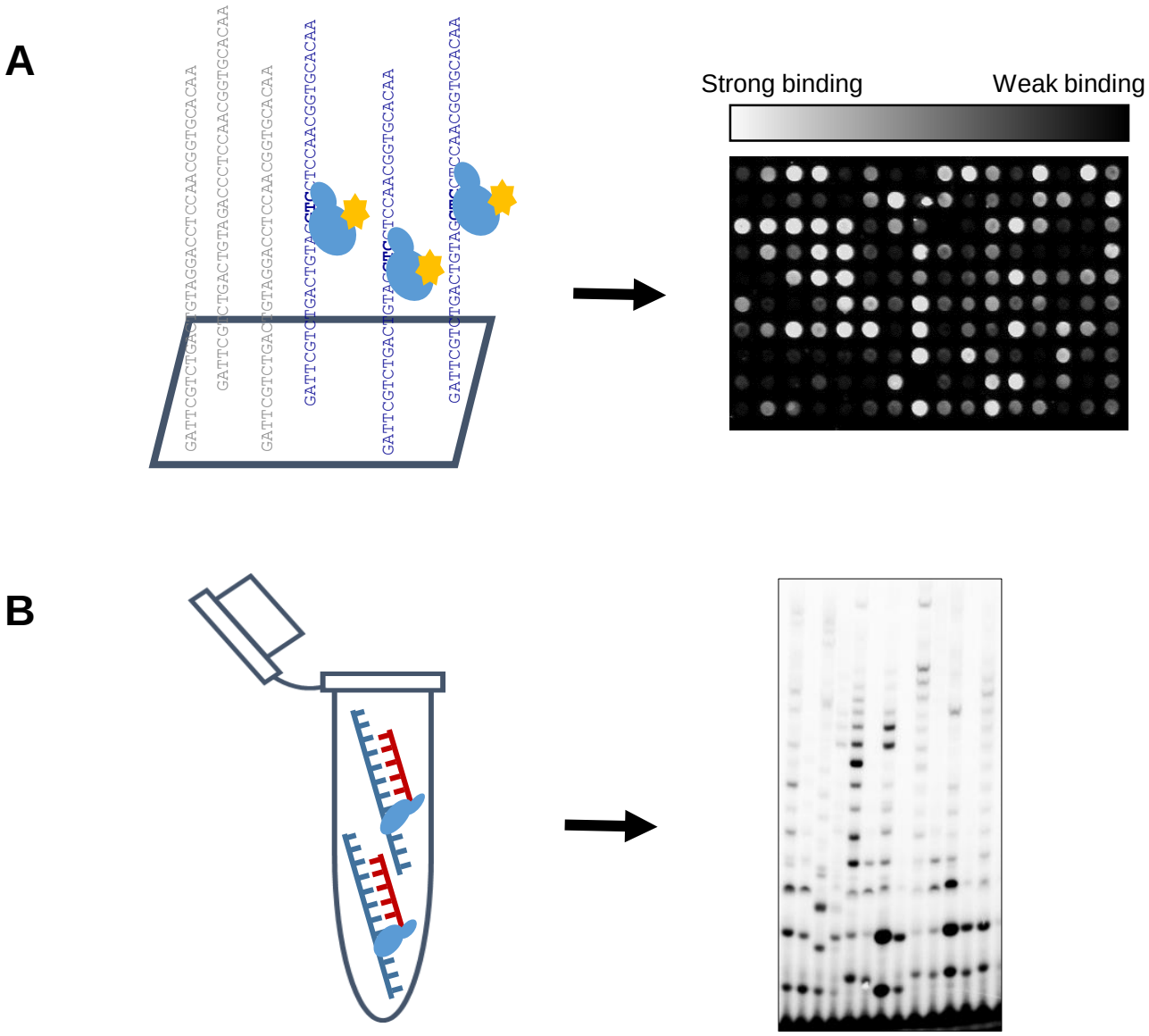
Figure 1



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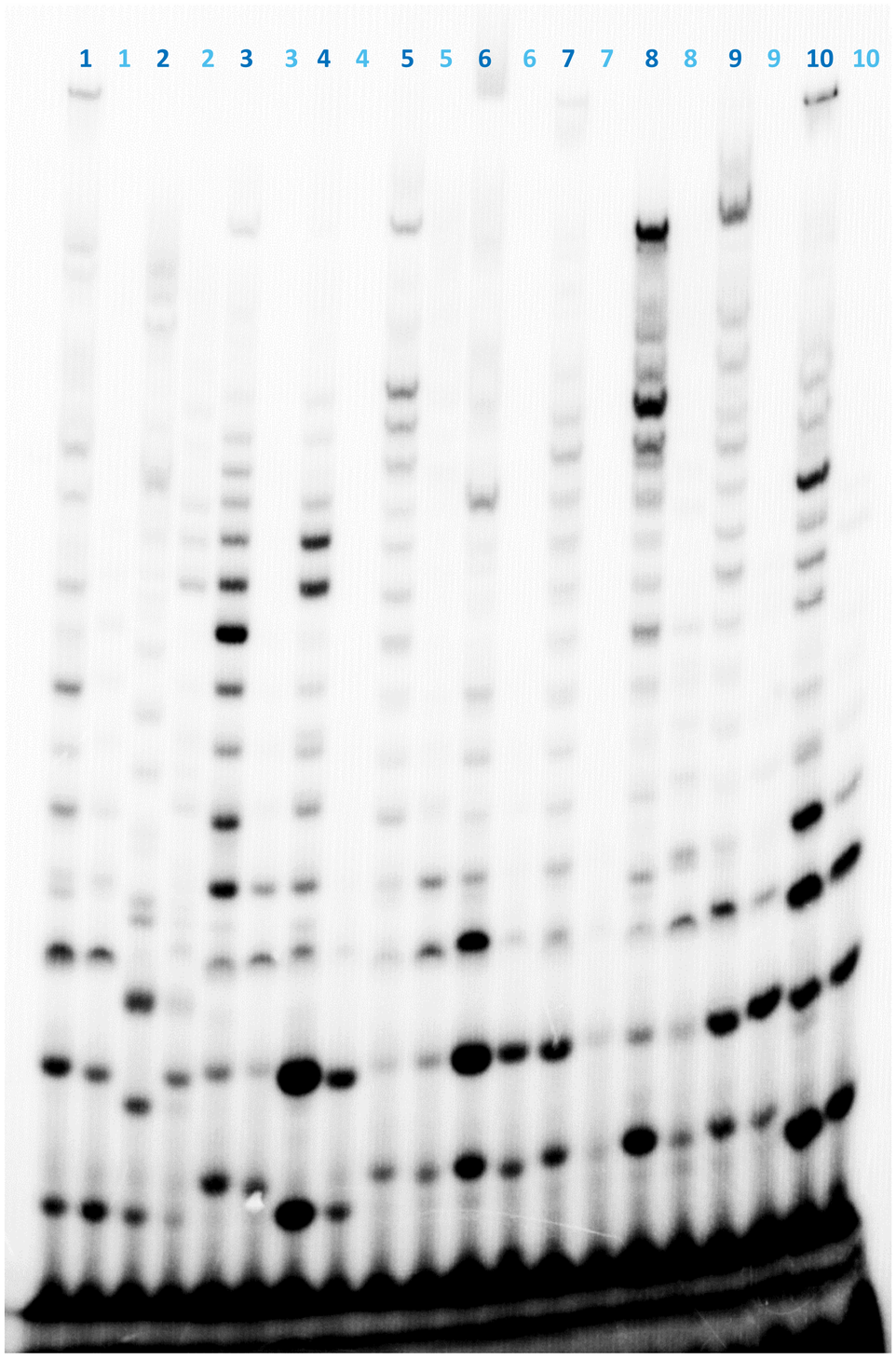

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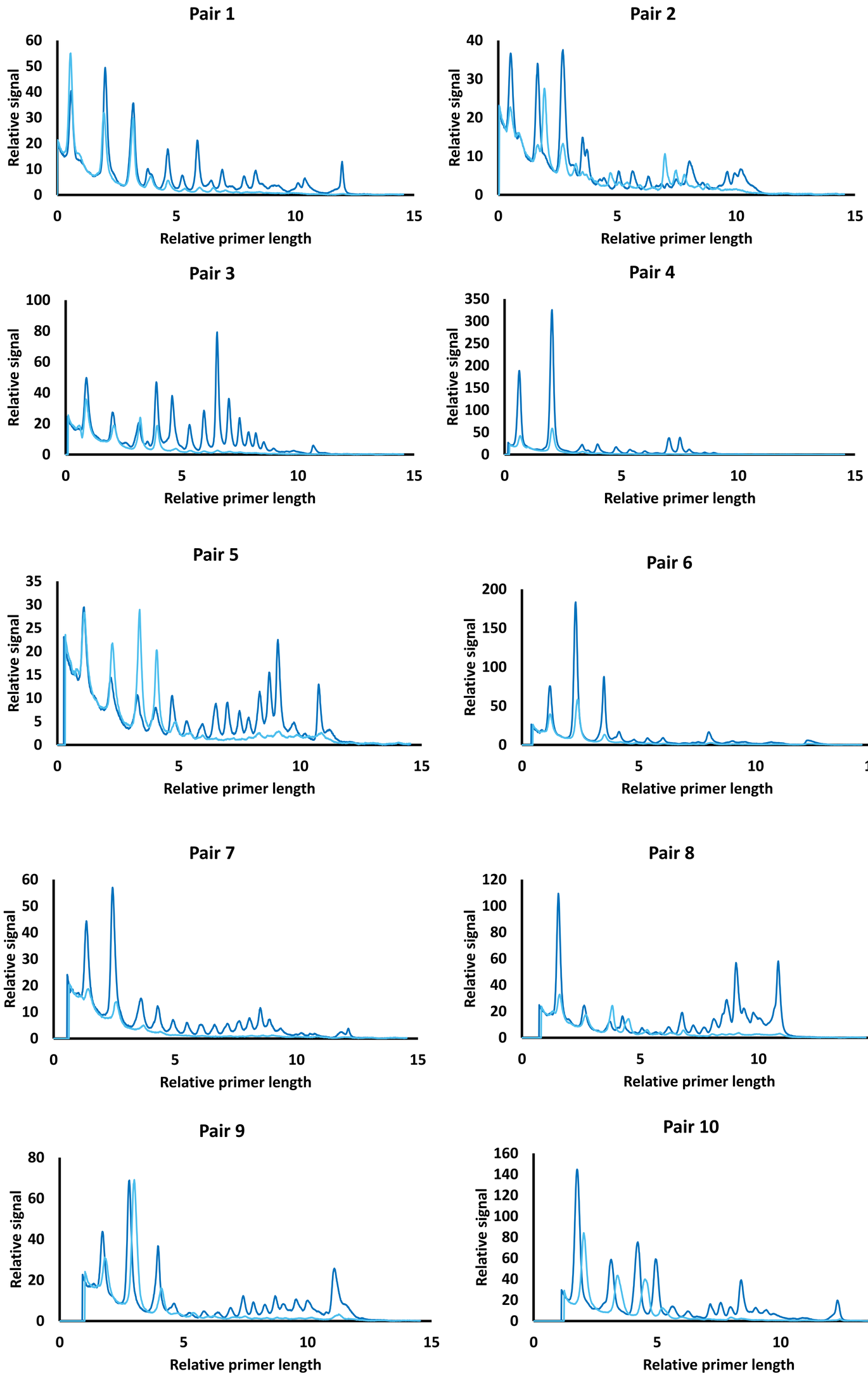

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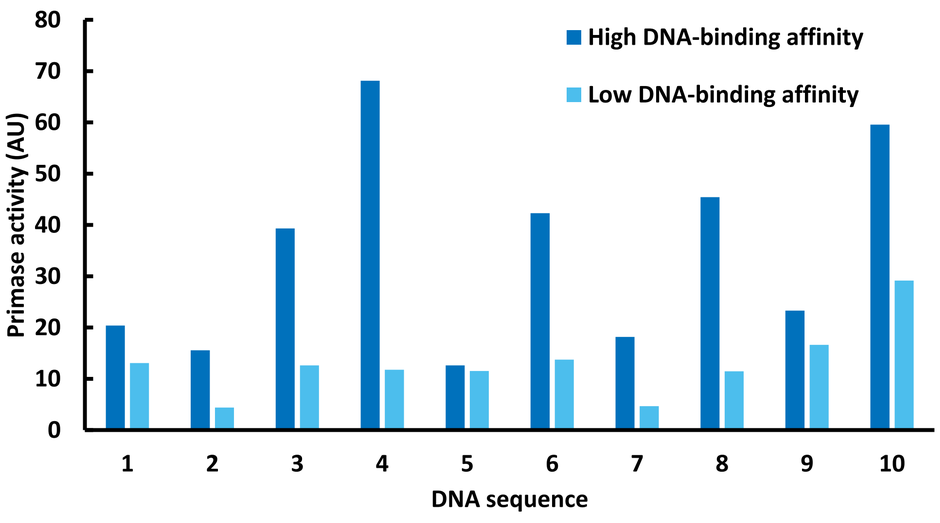

Material	Company	Catalog Number	
40% acrylamide-bisacrylamide (19:1) solution	Merck	1006401000	
95% formamide	Sigma-Aldrich	F9037-100ML	
Alexa 488-conjugated anti-his antibody	Qiagen	35310	
Ammonuium persulfate (APS)	Sigma-Aldrich	A3678-100G	
ATP, [α-32P] – 3000 Ci/mmol	Perkin Elmer	NEG003H250 UC	
Boric acid, granular	Glentham Life Sciences	GE4425	
Bovine Serum Albumin (BSA)	Roche	10735094001	
Bromophenol blue	Sigma-Aldrich	B0126-25G	
Coplin jar			
Dithiothreitol (DTT)	Sigma-Aldrich	D0632-25G	
DNA microarray	Agilent		4x180K (AMADID #78366) https://www.agilent.com
Ethylenediaminetetraacetic acid (EDTA)	Acros Organics	AC118430010	
Fujifilm FLA-5100 phosphorimager	FUJIFILM Life Science		
Glass slide staining rack	Thermo Scientific	12869995	If several slides are used
Lab rotator	Thermo Scientific	88880025	
Magnesium chloride	Sigma-Aldrich	63064-500G	
Microarray Hybridization Chamber	Agilent	G2534A	https://www.agilent.com/cs/libra
Microarray scanner (GenePix 4400A)	Molecular Devices		

Phosphate Buffered Saline (PBS)	Sigma-Aldrich	P4417-100TAB
Potassium glutamate	Alfa Aesar	A172232
Ribonucleotide Solution Mix (rNTPs)	New England BioLabs	N0466S
Salmon testes DNA	Sigma-Aldrich	D1626-1G
Skim milk powder	Sigma-Aldrich	70166-500G
Staining dish	Thermo Scientific	12657696
Tetramethylethylenediamine (TEMED)	Bio-Rad	1610800
Tris base (2-Amino-2-(hydroxymethyl)-1,3-propanediol)	Sigma-Aldrich	93362-500G
Triton X-100	Sigma-Aldrich	X100-500ML
Tween-20	Sigma-Aldrich	P9416-50ML
Urea	Sigma-Aldrich	U6504-1KG
Xylene cyanol	Alfa Aesar	B21530

ry/usermanuals/Public/G2534-90004_HybridizationChamber_User.pdf

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Author(s):	Stefan Ilic, Shira Cohen, Ariel Afek, Raluca Gordan, David B. Lukatsky, and .Barak Akabayov

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Rebuttal Letter:

We would like to thank the editor and the reviewers for very insightful comment and we hope that we have managed to address all of the raised issues as described in the following text. We have modified the manuscript, figures and figure legends to present the method more clearly, and in more detail, as required.

Editorial Comments:

• **Protocol Language:**
1) Please add steps under the subheading 1. At least 2 steps should be present under each subheading. Format of titles (steps) was changed to subheading.
2) Avoid listing reagents required in the protocol. Use the table of materials instead.
A list of reagents was deleted from the manuscript a table of materials is added instead.

• **Protocol Detail: Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps where needed. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.**
More details were added and corrections were made according to the reviewer's comments as presented in the following text.

• **Protocol Numbering: There must be a one-line space between each protocol step.**
Spacing was added.

• **Protocol Highlight: Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized.**

• **Discussion**. **Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol**.
The following text that covers the required topics has been added to the discussion:

1) modifications and troubleshooting,
"It is important to note that different primases require modifications of both microarray-binding conditions and buffer conditions for activity assays. For example, primase of Mycobacterium tuberculosis requires substitution of magnesium with divalent manganese in the reaction buffer. Inappropriate metal cofactor or inappropriate reaction buffer may lead to poor primase activity or decreased DNA-binding affinity. In addition, primases that bind to DNA templates with weak interaction require gentle washing steps during microarray experiment. Otherwise, they will detach from the microarray slide leading to partial or complete loss of florescent signal upon the addition of fluorescently labeled antibody."

2) limitations of the technique,
"On the other hand, the application of this method is limited by relatively high price of microarray slides as well as the safety precautions required when handling radioactive material used for primase activity assays. "

3) significance with respect to existing methods,
Significance was emphasized in several places. The following text was also added:
"To summarize, the DNA binding properties of many prokaryotic primases (including the trinucleotide DNA recognition sequence) are still poorly understood, mostly due to the technical limitations of currently available methods. HTPP represents fast and efficient platform for achieving this goal."
4) future applications
The following text was added:
"In addition, future application may be directed towards investigation of effect of different proteins or cofactors on DNA-binding affinity, recognition pattern or functional activity of primases and other DNA-processing enzymes. "
5) critical steps within the protocol
"Mild washing steps are critical, since the long washes with solutions containing detergents cause dissociation of the protein from the DNA template due to the weak/transient mode of binding. Other critical steps include binding conditions and incubation times that need to be optimized for each specific enzyme. "

• **References:** Please **do not abbreviate journal titles**. Corrected

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Corrected

• **Table of Materials:** Please **sort the items in alphabetical order** according to the name of material/equipment.
The table was corrected accordingly.

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The citation was added to both figures.

Reviewer #1:

Major Concerns:

My main concern here is that more than one primases should have been used to demonstrate wide applicability of this method. The T7 primase data are encouraging but is this particular primase more suited to this method than other primases? Can you demonstrate wider applicability?

The study was initially performed with T7 DNA primase and then it was extended to full gp4 protein (primase/helicase). We have also applied the same technique to investigate DNA-binding properties and to discover the trinucleotide recognition sequence of DnaG primase from *Mycobacterium tuberculosis* where we demonstrate wider applicability of the method (unpublished data).

Minor Concerns:

Is the T7 primase the combined helicase-primase or just the primase?

We have tested both the primase fragment and full primase/helicase gp4 protein (not presented in the manuscript for the sake of simplicity).

Would the binding site specificity be affected by the helicase-primase interaction?

We haven't tested the binding specificity of full gp4 protein by microarray. However, in biochemical assays we have observed the same activity pattern as for the primase fragment. Therefore, we have concluded that the helicase doesn't affect the binding specificity.

Line 97: change "costume" to "custom"

Corrected

page 203: Correct "ribnucleotide" to "ribonucleotide"

Corrected

Throughout the text all % values should be specified as w/v or v/v, as appropriate.

The values were specified accordingly.

Reviewer #2:

Manuscript Summary:

The methodological manuscript describes how to perform DNA binding experiments with T7 primase. The authors propose that the approach will help to "statistically identify specific features of DNA templates that affect the enzymatic activity of primase." It is not clear what is meant by this declaration, how the statistics is performed and why it is significant.

The more detailed statistical analysis is presented in section 3:

"All data processing was performed using custom written scripts in MATLAB. The initial PBM data processing is performed using Wilcoxon rank sum test p-value as described before. The median value of the binding intensity is then taken for each DNA sequence for further analysis. Next, using the one-way ANOVA p-values, we compare statistical significance of the observed differences in primase-DNA binding intensities obtained for different groups of DNA probes, as explained above in the DNA library design section. "

For example, how the DNA microarray should be designed to apply to other primases that do not have a strict recognition site?

The following paragraph covering this subject has been added to the discussion:

"To summarize, the DNA binding properties of many prokaryotic primases (including the trinucleotide DNA recognition sequence) are still poorly understood, mostly due to the technical limitations of currently available methods. HTPP represents fast and efficient platform for the discovery of the DNA recognition site or investigation of other template-related factors (overall composition, repeats, symmetry) that affect the binding affinity and activity of ssDNA-binding enzymes. In addition, future application may be directed towards the effect of different proteins or cofactors on DNA-binding affinity, recognition pattern or functional activity of primases and other DNA-processing enzymes."

Major Concerns:

Essentially, the manuscript attempts to provide some details of experimentation that are already present in the interesting iScience paper published a year ago. Many sentences and even illustrations are directly taken from the previous publication (e.g., compare the gel on the right part of Fig 1 of the current manuscript and the gel image in Fig 1D of the iScience paper). It is not clear why it is important to duplicate the information in additional paper.

The figure has been changed to add additional aspects of the methodology.

It remains unclear what conclusions are derived from "massive amount of data" because no analysis of the results with around 30,000 sequences is described (just examples are shown), while the enzymatic activity is tested only for a few sequences.

Enzymatic activity is the "standard way" to evaluate recognition sequence of a primase. Because it is limited in the amount of data it provides it serves as a validation phase after PBM where "massive" binding data for primase is first evaluated. Representative data for enzymatic activity is presented for 10 pairs of DNA template.

The results with control sequences with no 5'GTC site or with no primase would be interesting to see to understand the contribution of non-specific binding.

The following sentence was added to section 3: "Control oligonucleotides that didn't contain 5'-GTC-3' were also used. " The following sentence was added to the results section: "No primase binding was detected to DNA templates that were missing 5'-GTC-3' within their sequence."

It is also not clear if the claim that the primase that binds more tightly has elevated processivity is based on single-hit conditions of the reaction. Unfortunately, the current submission confuses the reader.

Initial studies with T7 DNA primase were performed with 1:3 primase-DNA ratio. However, when the study was extended to full gp4 protein 1:40 ratio was used (single-hit conditions) and the same pattern was observed.

The most critical steps of the protocol are described lightly (design of oligonucleotides, making the arrays, types of controls needed, the steel "PBM" chamber, what platform is used for mixing), while the authors describe in unnecessary precision how to make PAAG gels, the common knowledge.

Design of oligonucleotides: More details were added to section 1.

Making the array: The array is commercial. Therefore, we don't have the information regarding the manufacturing procedure.

Types of controls needed: Section 1.1.5 was added to describe the control oligos.

The term "steel PBM chamber" has been changed to "steel hybridization chamber (PBM chamber)" and the details were added to the table of materials.

The platform used for mixing is a lab rotator, and this information has been added to the protocol.

Minor Concerns:

Line by line comments.

Text:

52. Explain what is "statistically identify".

This has been explained in more detail in section 3.

53. Explain what "knowledge leap" is achieved.

For the first time we have shown a link between the DNA binding affinity and functional activity (processivity) of important DNA-processing enzyme.

67-68. The meaning of the sentence is difficult to understand.

The sentence has been rephrased: In particular, the important link between primase-DNA binding preferences, modulated by DNA sequences flanking specific tri-nucleotide binding sites, and the primase processivity, has been identified for this enzymatic system.

73. Reveal, what "unprecedented insight" was achieved.

The composition of the sequences flanking the known trinucleotide recognition sequence affect the primase binding affinity, resulting in different functional activity.

80. Are the authors sure about "Southern blotting"?

Corrected to southwestern blotting.

89-90. So far, the reader sees that the "unprecedented power" resulted in the only two classes of low binding and high binding sequences.

We have also identified intermediate DNA-binding affinity. However, we have focused on the two groups of DNA templates (week-binding and strong-binding) hoping to observe more distinctive effects on functional activity.

196. Molar concentration should be given.

Values were changed to molar concentrations.

275-276, 279. The idea of increased processivity is not proven, in the absence of the info on how single-hit conditions were met.

These conditions were met with full gp4 protein (not presented in this manuscript) as described above.

Figures:

Fig 1.

a. Why AAC sequences are in bold? Note that in similar Fig 1B in iScience paper GTC sequences are in bold. Changed to GTC.

b. The flow diagram creates an impression that the binding and activity experiments are directly connected, while they are not. Only a few sequences are used for the activity, in a different experimental setup. The figure has been changed accordingly.

Fig.2

a. How are "pairs" defined and selected? Do the numbers in panel A. correspond to these pairs as in panel C? Why ten pairs are shown, do they represent specific classes?

Text in Figure 2 and results were modified for clarification: …"two groups of sequences were identified: strong-binding DNA sequences that contained T/G in the flanks and weak-binding sequences that contained A/G in the flanks (all thymines in the strong-binding templates were

replaced by adenines). Ten pairs were chosen that present the best binding values to primase. “

b. What is the primase activity unit, what is "AU" in panel C?

The following statement was added to Figure 2: “AU (arbitrary unit) is the measure of intensity of radioactive signal which directly correlates with the amounts of synthesized RNA primers. “

c. Typo in "relative". What is relative primer length? What is relative signal?

Fixed.

The term relative indicates that the number of ribonucleotides constituting each RNA primer is unknown. This statement was added to Figure 2 legend: Relative length (number of ribonucleotides constituting each RNA primer is unknown) and amount of synthesized RNA primers by T7 DNA primase.

Reviewer #3:

Manuscript Summary:

Describes a method for measuring the binding affinity of T7 primase to a large collection of ssDNA sequences, using PBM technology. It then separately measures amount and length of primers made for a small subset of those sequences, chosen to highlight specific feature differences between low and high affinity sites. The results about the effects of context on primase binding and activity are interesting.

Major Concerns:

none

Minor Concerns:

they highlight that this is a high-throughput method, which is true for the binding assays. but for the primer length and quantitation it uses standard (i.e. low throughput) EMSA technique, which is clear once you've read it but that seems to be glossed over in the abstract/intro.

The biochemical assays serve to validate results we obtain from high-throughput microarray binding assay. Overall, the method enables us to rapidly identify a small group of DNA templates with interesting features and examine their effect on functional activity of DNA primase.

Reviewer #4:

Manuscript Summary:

This manuscript presents the protocol for HTPP (high-throughput primase profiling), an in vitro assay capable of measuring the DNA binding and the catalytic properties of DNA primase in a high-throughput manner. The assay overcomes the limitations of the conventional methods, including the characteristics of low-throughput and the failure of detecting low-affinity binding, thus allowing to reveal the specific binding mechanism of DNA primase and insights into the behavior of primer synthesis through a large-scale analysis.

The protocol introduced here is clearly explained and can be helpful for other potential applications or later extensions to a variety of enzymes. Overall, this is an important work worth publishing; however, the manuscript in the current form provides insufficient information in some places, possibly hindering the reproducibility and concept delivery of the experiment. I suggest the authors should clarify the following concerns and look forward to seeing this work getting published.

Major Concerns:

1. The authors mention that HTPP has the ability to measure low-affinity bindings of DNA primase comparing to the other conventional methods such as PBM. However, I don't see the detailed explanation of how this can be done in the sections of Introduction and Protocol. Even though the authors in the Discussion have mentioned the mild washing step, it still unclear whether this is the main factor allowing to detect low-affinity bindings or there are other factors, such as specific incubation conditions, buffer conditions, etc. of the protein and the antibody. The authors should emphasize this in the section of Protocol.

This segment has been added to the Discussion: "Mild washing steps are critical, since the long washes with solutions containing detergents cause dissociation of the protein from the DNA template due to the weak/transient mode of binding. Other critical steps include binding conditions (e.g. buffer composition, cofactors) and incubation times that need to be optimized for each specific enzyme. "

2. The authors solely provide a link for the commercial company, Agilent, where one can buy the microarray conducted in this experiment; however, the information provided is insufficient, which may cause confusion during the reproductive work. The authors should provide detailed information about the microarray used in the experiment such as the format and the size of the microarray.

We have added the following description to section 1: "DNA probes represent custom 36-nucleotide sequences, consisting of the recognition site for T7 DNA primase (GTC) located between two variable flanking regions, followed by a constant 24-nucleotide sequence tethered to a glass slide (Afek et al. iScience 2019). We used 4x180K microarray format which enabled spotting of each DNA sequence in six replicates, randomly distributed on the slide."

3. **The authors do not provide enough information on how they chose the sequences on the microarray. For example, do (N)17 and (N)16 share the same distribution (similar DNA composition) or not?**

Section 1.1.4 was added for more detailed describe of the sequences.

In addition, is there any specific reason for choosing 'GTC', the specific binding site for the recognition of the DNA primase, as the first triplet in the last 24 constant nucleotides? DNA primase may have a chance to bind at this position. If so, how can the authors support the claim that this recognition is due to the flanking variation.

The general sequence of oligonucleotides on microarray was corrected to:
$GTCTTGATTCGCTTGACGCTGCTG(N)_{16}GTC(N)_{17}$
Please note that the first GTC in the constant region is attached to the glass slide and therefore the primase can't bind to it.

4. **The authors do not introduce any control to the experiment in the manuscript. The authors need to clarify.**

Control samples (DNA without 5'-GTC-3') were used in the microarray experiment and no binding was observed. The following sentence was added to the section 3: "Control oligonucleotides that didn't contain 5'-GTC-3' were used to detect non-specific binding.
The following sentence was added to the Results section: No primase binding was detected to DNA templates that were missing 5'-GTC-3' within their sequence. "

5. It would be much more formal and apt to provide the full name of a term before using its abbreviation in the first instance; for example, Phosphate Buffered Saline (PBS).
Terms names were corrected along the manuscript.

6. The authors do not explain why the mixture has been dived into seven plastic tubes in Step 4.1.2. The authors should explain this.
This has been clarified: "Divide the mixture into 14 mL aliquots (the amount needed for one gel of 16.5 cm x 26 cm x 0.3 cm) and keep them protected from light at 4 ^{o}C for up to one month. "

7. The authors mention two groups of DNA templates obtained from PBM in Figure 2, but do not explain what these groups represent. The authors should provide more information on this.
This has been clarified both in the figure title and the figure legend.

Minor Concerns:
1. In line 135 on page 3, 5 uM T7 DNA primase -> µ
Corrected

2. The authors do not do punctuation well. For example, there are plenty of missing periods in the end of the sentences.
Corrected

Reviewer #5:

Major Concerns:
I have no major concerns.

Minor Concerns:

1. The manuscript needs copy-editing for grammar, sentence construction and language. Notable are (i) the sentence beginning at line 63 is confusing,
Rephrased to: Such high-throughput approach allows us to determine, in a systematic, quantitative, and hypothesis-driven way, the sequence-related properties that are important for primase binding and its enzymatic activity
(ii) "costume" should be "custom" at line 97, Corrected
(iii) delete "add the array" at line 146, Deleted
(iv) ribonucleoside triphosphate at line 203, Corrected
(v) phosphorimaging plate at line 222. Corrected

2. The running buffer for gel electrophoresis is not described. Added to steps 4.3.1 and 4.3.2.

3. Figure 2: "relative" not "realitive". Corrected
It is also not clear what "relative primer length" actually means - relative to what? Surely each peak in the scans corresponds to progressively longer primers, so the actual lengths can be counted.

This has been clarified in the figure legend: “Relative length (number of ribonucleotides constituting each RNA primer is unknown) and amount of synthesized RNA primers by T7 DNA primase. “

The reader is also unclear from the legend what are the template pairs in two different shades of blue both in the gel and the scans - how do they differ, in a general way?

This has been clarified both in the figure title and the figure legend.

Further comment: This comment does not necessarily impact on this methods manuscript, but does impact on the interpretation of data here and in reference 3. The composition of the ssDNA microarray described at line 101 (and in ref. 3) appears to show a design error in that the 5'-GTC-3' primase recognition site at which primer synthesis begins actually occurs twice in each oligo, i.e. between the two variable regions (as designed) and at the beginning of the 24-Nt spacer region. It is unclear why a primase monomer would not bind at either (or both) of these sites, but this does not apparently lead to a high background in the microarray data or heterogeneity in primer synthesis. A possible explanation for this conundrum is that primase actually binds to both sites separated as they are by ~20 Nt, as a dimer, but primers are synthesized only from the distal site. If this could be tested, it could comment on the speculation in literature that bacterial primases actually function as dimers. It might also explain the lower than expected frequency of primer synthesis/utilization during DNA replication (i.e. the recognition site would now be two GTCs separated optimally by a certain distance, which might correspond better to observed Okazaki fragment lengths). It would also explain a puzzling aspect of this work; i.e. how it is that primase binding to a single recognition site, which is known to be weak and transient, can survive the washing steps used in the microarray detection protocol. Much stronger binding of a dimer to both sites would explain this. What is the effect of eliminating one or other of the GTCs on either the microarray data or primer synthesis?

The general sequence of oligonucleotides on microarray was corrected to:

$GTCTTGATTCGCTTGACGCTGCTG(N)_{16}GTC(N)_{17}$

Therefore, the first GTC in the constant region is attached to the glass slide and the primase can’t bind to it.