Electronic Supplementary Material (ESI) for Analyst.

Supplementary information

Multiplex detection of the big five carbapenemase genes using solid-phase recombinase polymerase amplification

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Experimental Details

Synthetic plasmids as template DNA for RPA reactions and LOD analysis. For limit of detection (LOD) analysis synthetic plasmids containing bla_{KPC-2}, bla_{OXA-48}, bla_{NDM-1}, bla_{VIM-2}, or bla_{IMP-1} gene sequences were synthesised by Integrated DNA Technologies (IDT). Plasmids were propagated in Top10 chemically competent *E. coli* cells (Novagen) and purified using the Pureyield Plasmid Midiprep System (Promega). Plasmid concentrations were calculated using the Qubit dsDNA BR Assay kit (Qubit).

Primer pairs against the big five carbapenemases. Sequences for the variants of bla_{KPC}, $bla_{OXA-48-like}$, bla_{VIM}, bla_{NDM}, and bla_{IMP} genes were downloaded from the Comprehensive Antibiotic Resistance Database (CARD) [1]. The searches retrieved a total of 46, 41, 39, 68, and 78 variants for bla_{KPC}, $bla_{OXA-48-like}$, bla_{NDM}, bla_{VIM} and bla_{IMP}, respectively. The coding regions of each of the variants retrieved for the big five carbapenemase genes were aligned and visualised (Jalview). Primer pairs for each of the big five were designed against conserved regions. The primer design parameters were as follows: size setting of 18-30nt, product size of 45-250 bp, GC content of 20-70% and a Tm value of 50-95˚C. The propensity of primers to form secondary structures in the form of primer dimers (homodimers and heterodimers) and hairpin formations were predicted using the Oligoanalyzer software provided by IDT [2].

RPA primer Screen. TwistAmp basic kits (TwistDX) were used for the primer screen. Reactions using the TwistAmp basic kit were carried out as per the manufacturer's instructions. Briefly, per reaction, 2.4 µL of 10 µM forward primer, 2.4 µL of 10 µM reverse primer, 29.5 µL of rehydration buffer, 10 µL of 0.1 ng/µL template DNA, and 3.2 µL of dH_2O were added to the lyophilised reaction pellet. The solution was inverted to mix followed by the addition of 2.5 µL magnesium acetate before finally mixing and pulse centrifugation. Each reaction was incubated in a heat block at 37 °C for 20 min. Four minutes into the 37 °C incubation the reactions were mixed and pulse centrifuged before returned to the heat block for the remaining 16 min. After 20 min each reaction was stopped by placing the reaction at 4°C. No template control (NTC) reactions were set up as negative controls. NTC contain all RPA reaction components as per template containing reactions except the template is substituted for an equivalent volume of H_20.

Agarose gel electrophoresis and densitometry analysis of the RPA primer screen. The products of RPA reactions from the primer screen were separated by size on a 3% $_{(w/v)}$ agarose gel. Agarose was melted in 0.5x TBE buffer (Tris-boric acid-EDTA buffer) (47 mM Tris, 45 mM boric acid, 1.3 mM EDTA) with 0.01% $_{(v/v)}$ GelRed nucleic acid stain (Biotium) and gels were cast. RPA reaction (8 µL) was mixed with 4 µL of nucleic acid loading buffer and 5 µL from each mixture was transferred into separate wells of the agarose gel alongside an EZ load 20 bp molecular ruler (Bio-Rad) for molecular weight estimation and E-gel low-range quantitative DNA ladder (Bio-Rad) to facilitate quantification of DNA. Electrophoresis was performed at 100 V for 2 hr. An image of the resulting gel was captured using a ChemiDoc XRS+ (Bio-Rad). Densitometry was performed using Image lab software (Bio-Rad) in combination with the EZ load 20 bp molecular ruler and the E-gel low-range quantitative DNA ladder used as standards.

Generation of single stranded nucleotide tail sequences. A bespoke programme that combines Primer3 and NCBI nucleotide blast in tandem was created *in house*. The process performed by the script to generate six unique tails is detailed further in Figure S3.

Solution-phase RPA using tailed forward and reverse primers. TwistAmp basic kits (TwistDX) were used for solution RPA. Reactions using the TwistAmp basic kit were carried out as per the manufacturer's instructions. Briefly, per reaction, 2.4 µL of 10 µM forward primer, 2.4 µL of 10 µM reverse primer, 29.5 µL of rehydration buffer, 10 µL of template DNA, and 3.2 µL of dH_2O were added to the lyophilised reaction pellet. The solution was inverted to mix and centrifuged followed by the addition of 2.5 µL of 280 mM magnesium acetate to the lid and a second centrifuge step to initiate the reaction. Each reaction was incubated in a heat block at 37 °C for 20 min. Four minutes into the 37 °C incubation the reactions were mixed and pulse centrifuged before returned to the heat block for the remaining 16 min. After 20 min each reaction was stopped by placing the reaction at 4 °C. No template control (NTC) reactions were set up as negative controls. NTC contain all RPA reaction components as per template containing reactions except the template is substituted for an equivalent volume of H_20.

ELONA analysis of solution-phase RPA tailed forward and reverse primers. Streptavidin-coated microplates were washed three times with 200 µL of phosphate buffered saline + 0.05% $_{(v/v)}$ tween-20 (PBSt). Washed plates were then functionalised with 13.25 pmol of Biotin-TEG capture probe in 50 µL of phosphate buffered saline (PBS) and left to incubate overnight at 4 °C, covering the microplate with a plate seal. Three subsequent wash steps using 200 µL of PBSt were performed prior to transferring RPA reactions to each well and incubating at room temperature for 1 hr on a plate shaker set at 240 rpm. RPA reactions generated in solution using tailed forward and reverse primers were diluted 1 in 100 to a final concentration of 4x SSC (0.6 M NaCl, 0.06 M Sodium Citrate, 0.1% SDS $_{(w/v)}$, 1.4% Triton X-100 $_{(v/v)}$, and 5% formamide $_{(v/v)}$. After 1 hr incubation at room temperature in order to capture the RPA amplicons three wash steps with 200 µL of PBSt were performed. Next, 50 µL of 1 nM reporter probe diluted in 4x SSC was transferred to each well and incubated for 1 hr at room temperature on a plate shaker set at 240 rpm. Subsequently three wash steps were performed using 200 µL of PBSt. Finally 100 µL of TMB peroxidase substrate (KPL SureBlue Seracare) was added to the wells and the absorbance was measured at 630 nm using a benchtop microplate reader (Tecan infinite M200).

Resonance Raman Spectroscopy. TMB development was allowed to proceed before it was removed using a multichannel pipette and placed into a sterile microplate to stop the reaction. Samples were then scanned using a portable Raman spectrometer (Sierra, Snowy Range Instruments, USA). All the Raman measurements were carried out using orbital raster scanning (ORS) mode over the range 1000-1800 cm^{-1} using the Peak 1.3.68 software. A 638 nm laser excitation wavelength with 35 mW of laser power at source was used with an acquisition time of 5 s. The collected Raman spectra were then baseline corrected using MATLAB software. Finally, the average intensity of the Raman signal of the TMB Raman peak at 1611 cm^{-1} was plotted against a calibration data for each gene.

Supplementary Figures and Discussion

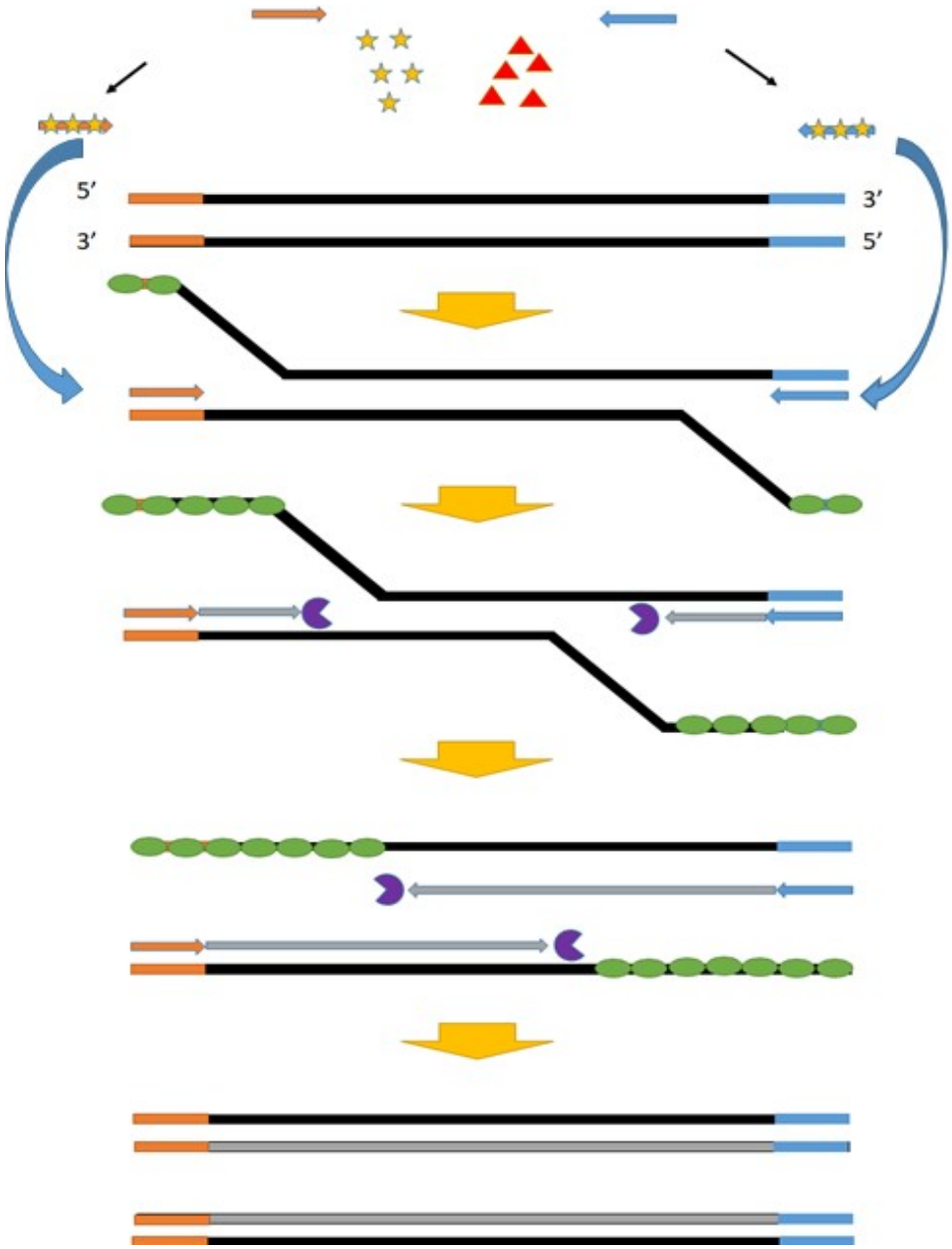

Figure S1: Schematic of RPA reaction mechanism. Loading factor (red triangles) promotes the loading of recombinase (gold stars) onto forward and reverse primers (orange and blue rectangles, respectively). These nucleoprotein filaments then scan the double stranded DNA for their cognate binding sequences. Once identified, the filaments invade the double stranded DNA, forming a D-loop structure to initiate a strand exchange reaction while single stranded binding protein (SSB) (green ovals) stabilised the displaced DNA strand. The recombinase disassembles to reveal the 3' ends of the primers and a strand displacing polymerase (purple sectors) extends the primers generating two double stranded DNA molecules.

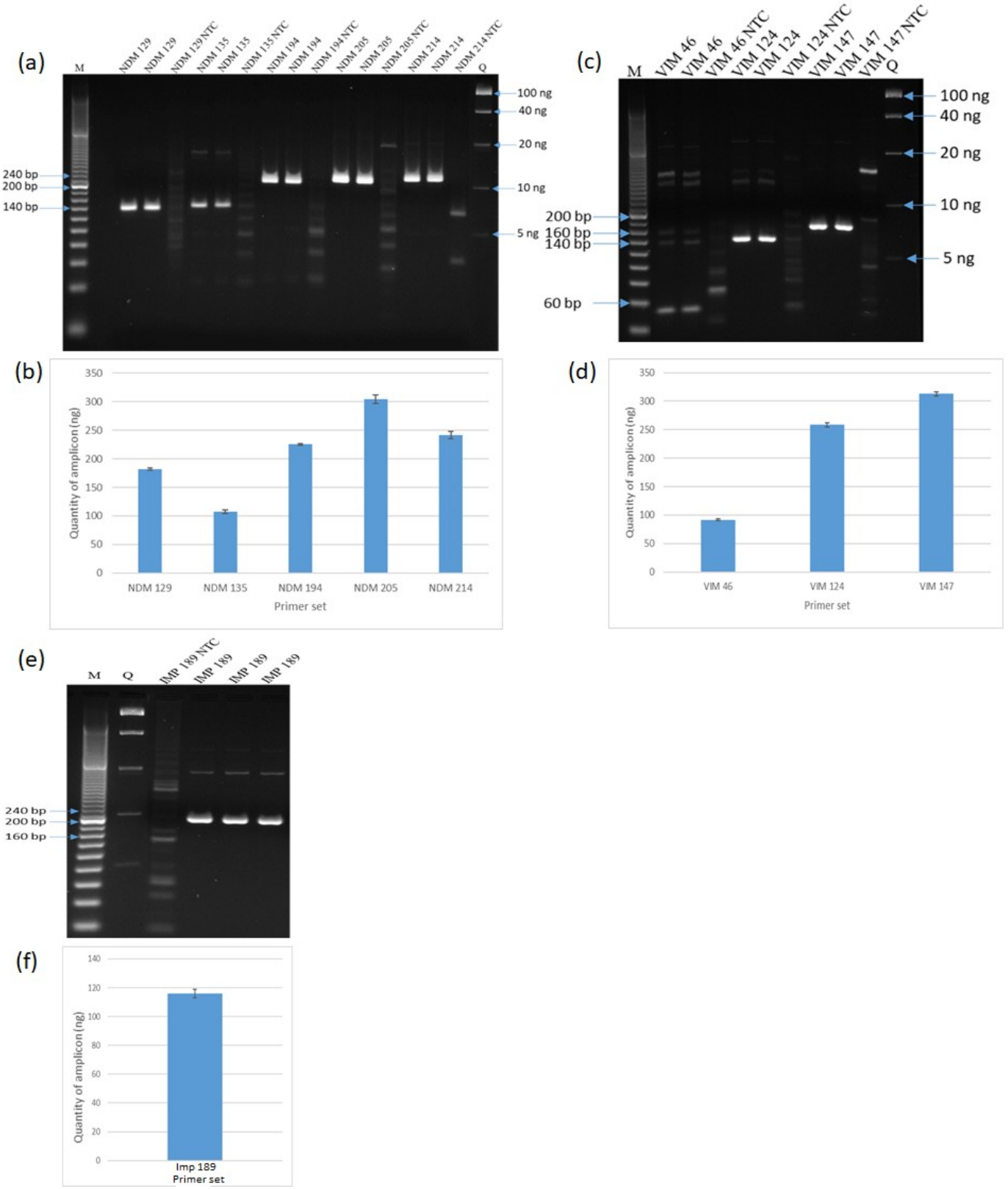
(a)
M
NDM 129
NDM 129
NDM 129 NTC
NDM 135
NDM 135
NDM 135 NTC
NDM 194
NDM 194
NDM 194 NTC
NDM 205
NDM 205
NDM 205 NTC
NDM 214
NDM 214
NDM 214 NTC
Q
100 ng
40 ng
20 ng
10 ng
5 ng
240 bp
200 bp
140 bp
(c)
M
VIM 46
VIM 46
VIM 46 NTC
VIM 124
VIM 124
VIM 124 NTC
VIM 147
VIM 147
VIM 147 NTC
Q
100 ng
40 ng
20 ng
10 ng
5 ng
200 bp
160 bp
140 bp
60 bp
(b)
Quantity of amplicon (ng)
350
300
250
200
150
100
50
0
NDM 129
NDM 135
NDM 194
NDM 205
NDM 214
Primer set
(d)
Quantity of amplicon (ng)
350
300
250
200
150
100
50
0
VIM 46
VIM 124
VIM 147
Primer set
(e)
M
Q
IMP 189 NTC
IMP 189
IMP 189
IMP 189
240 bp
200 bp
160 bp
(f)
Quantity of amplicon (ng)
140
120
100
80
60
40
20
0
Imp 189
Primer set

Figure S2: Agarose gel electrophoresis and densitometry analysis of NDM, VIM and IMP primer screens. (a) - Agarose gel electrophoresis (3%) of RPA reactions performed at 37 °C for 20 min using five different sets of primers targeted to the NDM gene (NDM 129, NDM 135, NDM 194, NDM 205, NDM 214). RPA reactions were performed in duplicate alongside a single NTC for each primer pair. Lane M – 20 bp molecular weight marker; lane Q - low range quantitation ladder (5-100 ng). (b) - Densitometry measuring the quantity of amplicon from an agarose gel electrophoresis loaded with RPA reactions containing 90 pM of NDM template DNA. Error bars represent the range between duplicates. (c) - Agarose gel electrophoresis (3%) of RPA reactions performed at 37 °C for 20 min using three different sets of primers targeted to the VIM gene (VIM 46, VIM 124, VIM 147). RPA reactions were performed in duplicate alongside a single NTC for each primer pair. Lane M – 20 bp molecular weight marker; lane Q - low range quantitation ladder (5-100 ng). (d) - Densitometry measuring the quantity of amplicon from an agarose gel electrophoresis loaded with RPA reactions containing 90 pM of Vim template DNA. Primer sets are named after the size of the amplicon they produce. Error bars represent the range between duplicates. (e) - Agarose gel electrophoresis (3%) of RPA reactions performed at 37 °C for 20 min using one set of primers targeted to the IMP gene (IMP 189). RPA reactions were performed in triplicate alongside a single NTC. Lane M – 20 bp molecular weight marker; lane Q - low range quantitation ladder (5-100 ng). (f) - Densitometry measuring the quantity of amplicon from an agarose gel electrophoresis loaded with RPA reactions containing 90 pM of IMP template DNA. Primer sets are named after the size of the amplicon they produce. Error bars represent the standard deviation of the triplicate reaction.

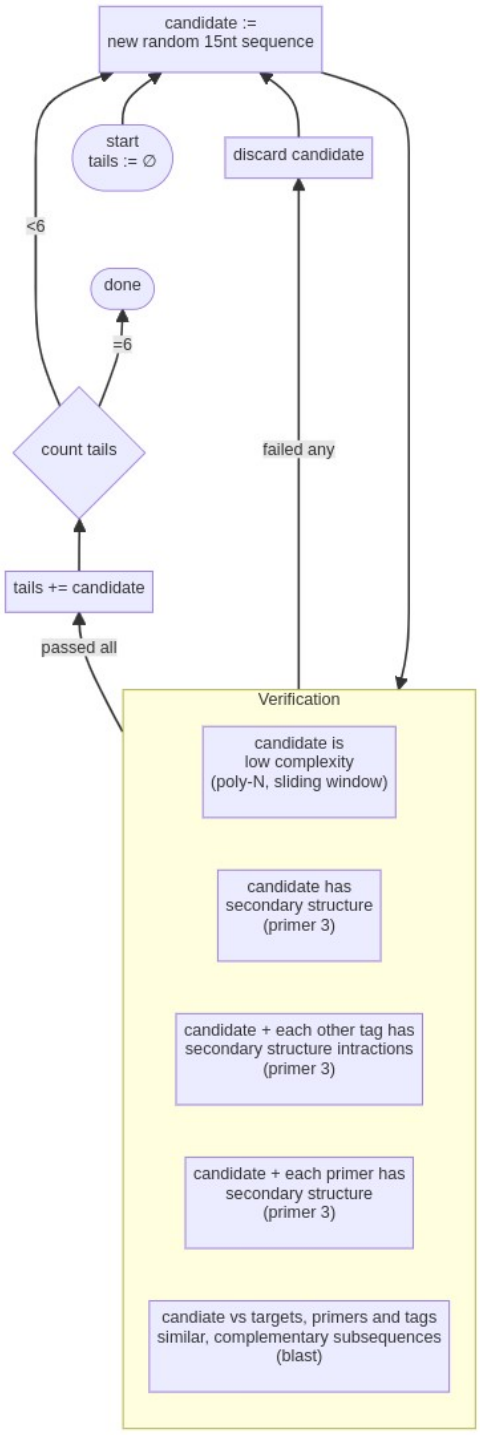

Figure S3: Flow diagram detailing the process of designing six unique oligonucleotide tail sequences. The algorithm begins with an empty set (Ø) of tails, and attempts to grow this set until the desired number are designed. In each round, a new pseudorandom candidate tail sequence is sampled from uniform distribution over nucleotides, within the required length range. This is then verified. If the candidate passes the verification process, it is added to the tails set (+=). If the tails set is of the required size (=6), the process terminates, reporting the tail set. If not (<6), it goes back to sampling a new candidate. If any of the verification steps fail with a candidate tail, it is discarded and a new one sampled to replace it. It is possible for this process to deadlock if a partial tail-set happens to make it unlikely for any new candidate to pass verification. To handle this, the algorithm will result in failure if too many candidates have been tried and rejected (not shown in the state diagram). Verification performs three categories of checks: the candidate against itself only, candidate against the current tail set and primers, and the candidate vs the RPA target sequences. Tails are checked for internal secondary structure and low-complexity regions (near-perfect poly-n runs). All secondary structure searches are performed using Primer3 and rejecting any sequences where it reports secondary structure above its default thresholds. The tail is then checked for secondary structure against all tags, and against all primers. To cover the edge case where it forms a secondary structure to the fusion region between a tag and its primer, it is also verified against all pairwise fusions of tags and primers. Lastly, the candidate is blasted (blastn) against the gene sequences for the RPA targets with the blastn defaults. Any candidate with any hits is discarded.

The software is available at: https://bitbucket.org/ncl-intbio/primer_tails

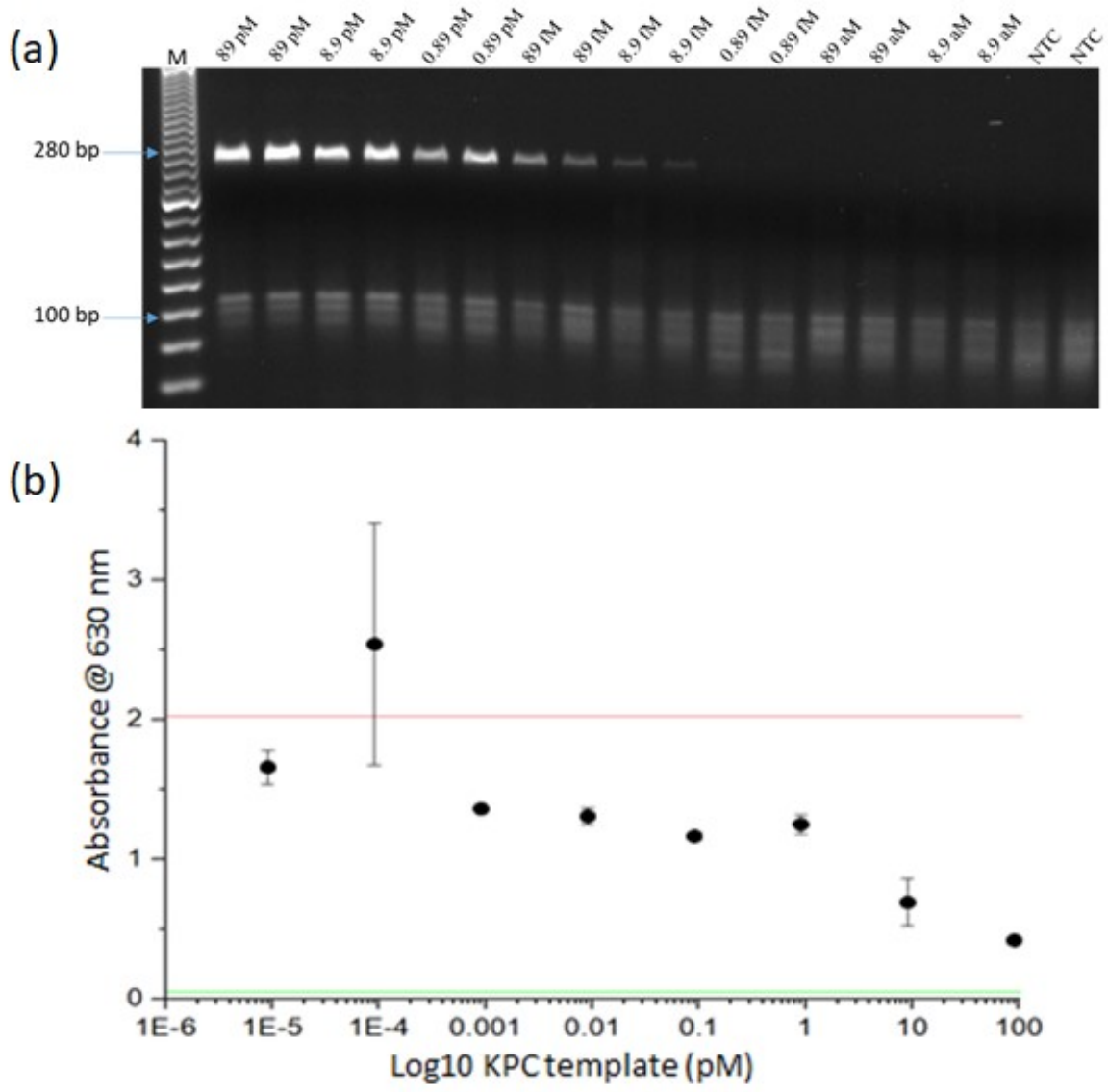

Figure S4: RPA in solution using tailed KPC 203 forward and reverse primers. (a) Agarose gel electrophoresis (3%) visualising the products of RPA reactions using tailed KPC 203 primers, across a range of template DNA concentrations (89 pM – 8.9 aM). (b) ELONA analysis measuring the absorbance at 630 nm of the same RPA reactions. Absorbance was measured 5 min after the addition of TMB substrate. Each point is the average of duplicates and error bars represent the range between duplicates. The mean absorbance of the NTC samples is represented as a horizontal red line and the average absorbance produced by buffer only is represented as the green line.

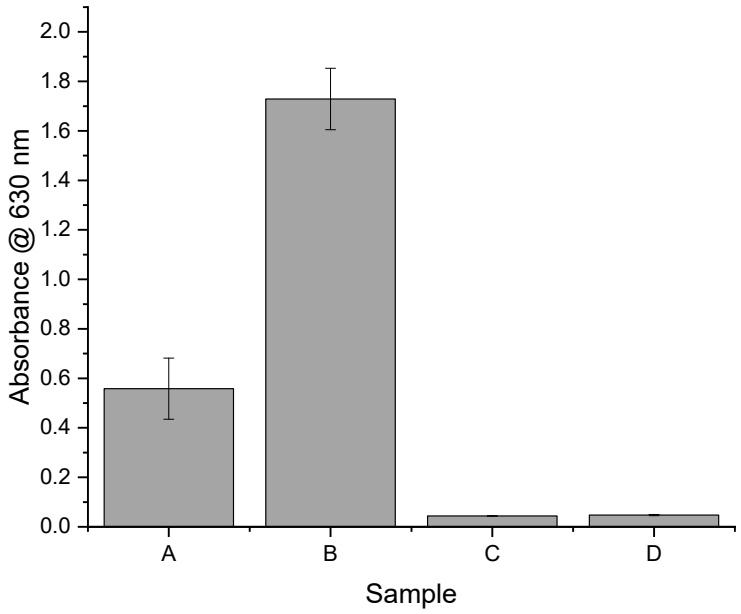

Figure S5: Bar chart assessing the cause of high background observed when carrying out solution-phase RPA combined with ELONA. An array of RPA reactions were assessed using ELONA. RPA reactions carried out are as follows, (A) OXA-48 template DNA, (B) reactions containing no template DNA (NTC), (C) reactions containing no template DNA (NTC) or primers, (D) reactions containing no template DNA (NTC) or magnesium. Each RPA sample was performed in triplicate and the products of each were transferred to an ELONA plate for analysis. Error bars represent the standard deviation between triplicates measurements.

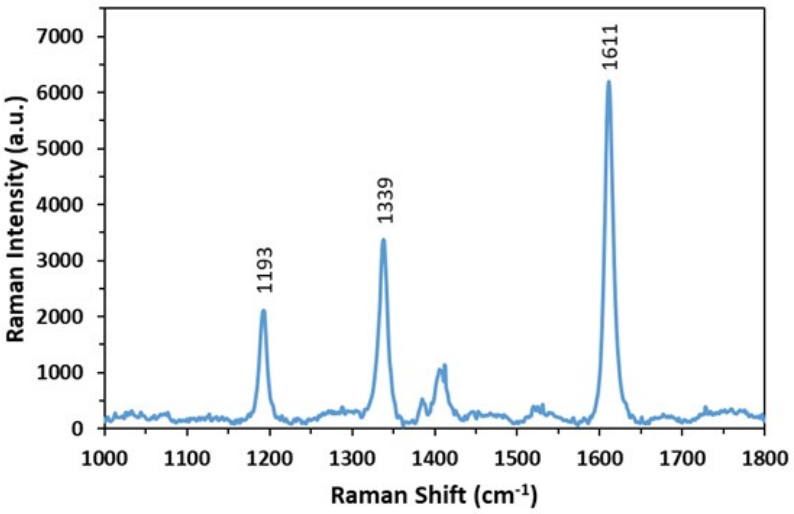

Figure S6. Resonance Raman spectrum of the oxidised TMB solution. All the resonance Raman measurements were carried out using a portable Raman spectrometer equipped with a 638 nm laser excitation source at 35 mW laser power with an acquisition time of 5 s at ORS mode.

Supplementary Tables

Primer name	Forward primer sequence	Reverse primer sequence
KPC 87	5′ CATCTCGGAAAAATATCTGACAAC 3′	5′ TTGGCGGCGGCGTTATCACTGTA 3′
KPC 114	5′ TTCTGCTGTCTTGTCTCTCATGG 3′	5′ CACCGATGGAGCCGCCAAAGTCC 3′
KPC 124	5′ GCTTTCTTGCTGCCGCTGTGCTGG 3′	5′ GTTGTCAGATATTTTTCCGAGATG 3′
KPC 204	5′ TACAGTGATAACGCCGCCGCCAA 3′	5′ GAGCCCAGTGTCAGTTTTTGTAA 3′
KPC 203	5′ AATACAGTGATAACGCCGCCGCC 3′	5′ CAGAGCCCAGTGTCAGTTTTTGTAAG 3′
OXA 155	5′ AAACAAGCCATGCTGACCGA 3′	5′ GTGGGCATATCCATATT 3′
OXA 186	5′ AACCAAGCATTTTTACC 3′	5′ TTGATAAACAGGCACAAC 3′
OXA 194	5′ TTAGCCTTATCGGCTGTGTT 3′	5′ GGTAAAAATGCTTGGTT 3′
OXA 239	5′ GTTGTGCCTGTTTATCAA 3′	5′ TCGGTCAGCATGGCTTGTTT 3′
NDM 129	5′ CAACTGGATCAAGCAGGAGATCAACC 3′	5′ GACAACGCATTGGCATAAGTCGC 3′
NDM 135	5′ ATCAGGACAAGATGGGCGGTATG 3′	5′ CATTGGCGGCGAAAGTCAGGCTG 3′
NDM 194	5′ CAACTGGATCAAGCAGGAGAT CAACC 3′	5′ CATTGGCGGCGAAAGTCAGGCTG 3′
NDM 205	5′ CGGTTTGATCGTCAGGGATGGC 3′	5′ CAACGCATTGGCATAAGTCGCAA 3′
NDM 214	5′ GCTGAGCACCGCATTAGCCGCTG 3′	5′ GCCATCCCTGACGATCAAACCG 3′
VIM 46	5′ ATGGTCTCATTGTCCGTGATGG 3′	5′ GTATCAATCAAAAGCAACTCATC 3′
VIM 124	5′ GATGAGTTGCTTTTGATTGATAC 3′	5′ GGTCGTCATGAAAGTGCGTGGAG 3′
VIM 147	5′ ATGGTCTCATTGTCCGTGATGG 3′	5′ GGTCGTCATGAAAGTGCGTGGAG 3′
IMP 189	5′ GGAATAGAGTGGCTTAATTCTCG 3′	5′ CCAAACCACTACGTTATCT 3′

Table S1: Primers designed against the big five targeting *bla*$_{KPC}$, *bla*$_{OXA-48-like}$, *bla*$_{VIM}$, *bla*$_{NDM}$, and *bla*$_{IMP}$ genes. The primer number indicates the size of the amplicon which will be produced when using this primer pair

Tail number	Tail sequence
1	5′ CTACATTACGAAGTT 3′
2	5′ GACCTGTGCTATCAA 3′
3	5′ CGAACCTTCCACTCG 3′
4	5′ TAGTTGGAACACCTT 3′
5	5′ GAAGACCCAACAGAG 3′
6	5′ TCAGGACACACTCTT 3′

Table S2: Generation of single stranded nucleotide tail sequences. Six tail sequences, each 15 nt in length were generated using an *in house* bioinformatics script.

Target gene	Capture probe	Tailed forward primer	Tailed reverse primer	Reporter probe
KPC	5' CTCTGTTGGGTCTTCTTTTTTTTTTTTTTT-TEG-Biotin 3'	5' GAAGACCCAACAGAG-C3-AATACAGTGATAACGCCGCCGCC 3'	5' TCAGGACACACTCTT-C3-CAGAGCCCAGTGTCAGTTTTTGTAAG 3'	5' HRP-AAGAGTGTGTCCTGA 3'
OXA-48	5' CGAGTGGAAGGTTCGTTTTTTTTTTTTTTT-TEG-Biotin 3'	5' CGAACCTTCCACTCG-C3-GTTGTGCCTGTTTATCAA 3'	5' TCAGGACACACTCTT-C3-TCGGTCAGCATGGCTTGTTT 3'	5' HRP-AAGAGTGTGTCCTGA 3'
NDM	5' AAGGTGTTCCAACTATTTTTTTTTTTTTTT-TEG-Biotin 3'	5' TAGTTGGAACACCTT-C3-CGGTTTGATCGTCAGGGATGGC 3'	5' TCAGGACACACTCTT-C3-CAACGCATTGGCATAAGTCGCAA 3'	5' HRP-AAGAGTGTGTCCTGA 3'
VIM	5' AACTTCGTAATGTAGTTTTTTTTTTTTTTT-TEG-Biotin 3'	5' CTACATTACGAAGTT-C3-ATGGTCTCATTGTCCGTGATGG 3'	5' TCAGGACACACTCTT-C3-GGTCGTCATGAAAGTGCGTGGAG 3'	5' HRP-AAGAGTGTGTCCTGA 3'
IMP	5' TTGATAGCACAGGTCTTTTTTTTTTTTTTT-TEG-Biotin 3'	5' GACCTGTGCTATCAA-C3-GGAATAGAGTGGCTTAATTCTCG 3'	5' TCAGGACACACTCTT-C3-CCAAACCACTACGTTATCT 3'	5' HRP-AAGAGTGTGTCCTGA 3'

Table S3: Sequences targeting the big five used for solution RPA with ELONA detection. Forward primer sequences are in red. Reverse primer sequences are in blue. 5 'single stranded tails sequences of the primers are in green. Biotin-TEG = Biotin tetraethylene glycol spacer, C3 = C3 spacer group, HRP = Horseradish peroxidase

Target gene	Forward primer	Tailed reverse primer	Reporter probe
KPC	5' Biotin-TEG-AATACAGTGATAACGCCGCCGCC 3'	5' TCAGGACACACTCTT-C3-CAGAGCCCAGTGTCAGTTTTTGTAAG 3'	5' HRP-AAGAGTGTGTCCTGA 3'
OXA-48	5' Biotin-TEG-GTTGTGCCTGTTTATCAA 3'	5' CGAACCTTCCACTCG-C3-TCGGTCAGCATGGCTTGTTT 3'	5' HRP-CGAGTGGAAGGTTCG 3'
NDM	5' Biotin-TEG-CGGTTTGATCGTCAGGGATGGC 3'	5' GAAGACCCAACAGAG-C3-CAACGCATTGGCATAAGTCGCAA 3'	5' HRP-CTCTGTTGGGTCTTC 3'
VIM	5' Biotin-TEG-ATGGTCTCATTGTCCGTGATGG 3'	5' GACCTGTGCTATCAA-C3-GGTCGTCATGAAAGTGCGTGGAG 3'	5' HRP-TTGATAGCACAGGTC 3'
IMP	5' Biotin-TEG-GGAATAGAGTGGCTTAATTCTCG 3'	5' TAGTTGGAACACCTT-C3-CCAAACCACTACGTTATCT 3'	5' HRP-AAGGTGTTCCAACTA 3'

Table S4: Sequences targeting the 'big five' used for solid-phase RPA with ELONA detection. Forward primer sequences are in red. Reverse primer sequences are in blue. Five prime single stranded tails are in green. Biotin-TEG = Biotin tetraethylene glycol spacer C3 = C3 spacer group, HRP = Horseradish peroxidase

References

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2 Owczarzy R, Tataurov AV, Wu Y, Manthey JA, McQuisten KA, Almabrazi HG, et al. IDT SciTools: a suite for analysis and design of nucleic acid oligomers. Nucleic acids research. 2008;36(Web Server issue):W163-W9.