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Clonal rolling circle amplification for on-chip DNA cluster generation

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Abstract

Generation of monoclonal DNA clusters on a surface is a useful method for digital nucleic acid detection applications (e.g. microarray or next-generation sequencing). To obtain sufficient copies per cluster for digital detection, the single molecule bound to the surface must be amplified. Here we describe ClonalRCA, a rolling-circle amplification (RCA) method for the generation of monoclonal DNA clusters based on forward and reverse primers immobilized on the surface. No primer in the reaction buffer is needed. Clusters formed by ClonalRCA comprise forward and reverse strands in multiple copies tethered to the surface within a cluster of micrometer size. Single stranded circular molecules are used as a target to create a cluster with about 10 000 forward and reverse strands. The DNA strands are available for oligonucleotide hybridization, primer extension and sequencing.

Keywords: isothermal amplification; forming DNA clusters; solid surface

Introduction

During the last decade, DNA amplification on a solid surface has become an important tool for digital DNA analysis, diagnostics, or next-generation sequencing methods [1, 2]. Several different methods have been established to generate DNA clusters by amplification methods. These methods include emulsion PCR (e.g. BEAMing digital PCR, 454 sequencing), bridge amplification (e.g. Illumina sequencing), rolling circle amplification (e.g. Complete Genomics) and amplification by template walking on the solid surface (SOLiD). In emulsion PCR, clusters are formed by a single template molecule bound to beads that are enclosed in small vesicles. The method involves generating emulsions, performing PCR, breaking emulsions, and enrichment of beads with a proper DNA cluster [3]. Single emulsion vesicles should not contain more than a single bead and a single target molecule. Because of Poisson distribution, high dilutions of beads and DNA target molecules have to be used for generating the emulsion. Statistically, only a minor fraction of all vesicles are suitable for downstream analysis and comprise a single cluster on a single bead. Therefore, an enrichment of beads positive for a cluster is necessary. After enrichment, most of the beads are clonal and contain the amplified DNA of a single target DNA molecule. Some of the beads comprise clusters of two different target molecules, depending on target DNA dilution and Poisson distribution. Despite the existence of instruments capable of automating this process, the method remains time-consuming and inefficient.

In order to circumvent the complex process of emulsion PCR, other technologies have been developed such as nanoball amplification which uses circular template molecules for rolling circle amplification (RCA). During nanoball amplification a

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specific primer hybridizes to the primer binding site and starts the linear RCA reaction. During RCA reaction, single-stranded DNA products are generated which fold as nanoballs. A single circular template molecule covers multiple fragments of a target sequence interrupted by adaptor sequences. Although standard RCA does not require hairpin structures for random coiling, for nanoball formation the adaptor sequences include short hairpin sequences required for coiling of amplified DNA by intramolecular folding. After RCA, nanoballs are bound onto a structured two-dimensional surface [4]. The generation of circular template molecules requires multiples rounds of adaptor ligation, purification, PCR, and circularization. Other methods for DNA circularization are much easier but do not introduce hairpin structures required for nanoball formation. In one example single-stranded target DNA is hybridized to another single-stranded DNA spanning the ligation site. After ligation a single-stranded DNA circle is formed [5–8]. In another example, double-stranded target DNA is used in a self-ligation reaction [9].

In contrast to emulsion PCR and nanoball generation, other methods singularize DNA target molecules by their spatial distribution on a two-dimensional surface prior to amplification. After binding to the surface, the target molecules are amplified. Several amplification methods have been developed to perform the amplification of DNA bound onto a surface. For bridge amplification [10], both amplification primers are immobilized to the solid support. After binding of a single-stranded DNA molecule to one primer, the primer is elongated to form the complementary strand. After denaturation and reannealing, the newly synthesized strands form a DNA bridge with another primer bound onto the surface. On automated platforms, the denaturation process during bridge amplification is performed by a chemical denaturation process. Therefore, every amplification cycle requires new reagents for DNA synthesis and denaturation. Multiple rounds of elongation, denaturation, and reannealing generate a cluster of multiple copies. Ma *et al.* [11] described an amplification process driven by template walking, also known as Wildfire amplification. The process starts by hybridizing template to primers bound to the surface. After primer elongation, the template undergoes a partial terminal denaturation (DNA breathing mechanism). The terminal denatured strand hybridizes to a primer bound in close vicinity (template walking). Next, the primer is elongated and forms a new copy by a strand displacement polymerase. In contrast to bridge amplification, only one primer sequence is immobilized onto the surface. The second primer is in solution and starts synthesis from the immobilized single-stranded DNA left over after DNA displacement. Because the displaced strand is not rebound specifically to the surface, it may form by-products in solution. Lizardi [6] described a RCA reaction on a glass surface for signal amplification. For this reaction, a primer is generated on a surface that starts a linear RCA reaction. Nallur described a similar technology for RCA and introduced a second primer for exponential signal amplification for microarrays. The second primer was included within the reaction solution to get the exponential RCA which is called L2RCA [12]. Although Nallur *et al.* described that a branched nucleic acid is primarily generated during L2RCA, strand displacement reactions (like RCA) may result also in a complete displacement of DNA strands. If using a homogeneous surface completely covered with the primers of the same sequence, displaced strands may form secondary DNA clusters in close vicinity affecting downstream applications (e.g. NGS).

Here, we describe ClonalRCA, a method that use circular DNA templates for surface bound exponential rolling circle amplification (RCA). In contrast to L2RCA, ClonalRCA circumvents the disadvantage of displacing DNA strands to other sites on the surface by extension of dissolved primers. In ClonalRCA, all primers are immobilized to the surface. In contrast to large DNA nanoballs, the small size of the circular DNA templates results in an uniform distribution of target DNA on the surface. Large DNA molecules are more sticky because first contacts, nucleation and stochastic zippings of single-stranded DNA molecules are a function of the length of the DNA [13]. Therefore, DNA nanoballs require short palindromic sequences within the circular target DNA to improve the compact shape of the RCA amplified DNA molecules [4]. No palindromic sequences are introduced for ClonalRCA templates because the compact shape of ClonalRCA clusters is obtained by extension of immobilized primers.

Material and methods

Circular Target DNA generation

Target-1 DNA (for sequence see Table 1) circularization is performed by hybridizing a complementary oligonucleotide followed by a ligation reaction using T4 DNA ligase (NEB, M0202S) according to the protocol provided by supplier. The CPP-library is started from PCR amplicons generated by the GeneRead DNAseq targeted Panel (QIAGEN, Cat.No. 181900) using the GeneRead DNAseq Panel PCR Kit (QIAGEN, Cat.No.181940). The PCR allows the amplification of ~600 different amplicons of 24 tumor relevant genes. The size of the PCR amplicons span from ~120 to 190 nt. A total of 10 ng of purified PCR fragments are used for library preparation using the GeneRead DNA Library l Core Kit (QIAGEN, Cat.No. 180432) and GeneRead DNA I Amp Kit (Qiagen, Ca.No. 180455). In contrast to standard adaptors, we use a different sequence for double-stranded left adaptor and right adaptor minimizing hairpin formation. Prior to circularization of the library, we performed the end-repair reaction (GeneRead DNA I Amp Kit, Qiagen, Cat.No. 180455). The end repair is performed for 30 min at 75 °C using the linear PCR fragments (5 ng/μl). The 75 °C inactivation step is avoided. For circularization the fragments range from ~200 to 280 bp (PCR fragment including left and right adaptor). Circle closure of dsDNA fragments is performed by a standard ligation reaction using the T4 DNA Ligase (NEB, M0202S). Ligation was performed for 60 min at 21 °C. In order to improve the formation of monomeric circles, we chose a very low concentration (0.5 ng/μl) of linear dsDNA fragments [9]. All oligonucleotides are listed in Table 1.

Neutravidin immobilization

For the immobilization of Neutravidin (Thermo Scientific, 31000) in GeneRead flow cells, a series of cover glasses are first treated in a solution of 2.5% (3-Aminopropyl)trimethoxysilane (Sigma, 281778) in 95% aq. ethanol for 30 min at 50 °C. After cooling down, the cover glasses are removed from this solution and immediately rinsed twice with 95% aq. ethanol then once with abs. methanol. The cover glasses are then dried in an oven at 110 °C. To reduce reagent consumption, the following reaction steps are done in the flow channel of the flow cell. In order to create the flow channel of the GeneRead flow cell, flow cell base and cover glass are bound tightly together by double sided adhesive gaskets. For the generation of neutravidin binding sites within the flow channel, each flow cell is filled with a 2 mM solution of NHS-PEG4-Biotin (Thermo Scientific, 21330) in DMSO

Table 1: Oligonucleotides used for ClonalRCA

Name	Sequence	Comment
Target-1 DNA	(5′) P-atg acg ata tga gtc aca ttt tgt tca tgg gca tga cat tga tac aca gtt aga cga tag gac agt aca ttc gac cta tcc ttg cgc agc tcg aga tga cg-OH (3′)	
Target -1 DNA complementary oligonucleotide	(5′) aaa tgt gac tca tat cgt cat cgt cat ctc gag ctg cg-OH (3′)	
Left adaptor	(5′) b-ccc tgt aca ctc aac caa ctt cac tca atc att cca tgc tac cca act c-OH (3′) (3′) b-ggg aca tgt gag ttg gtt gaa gtg agt tag taa ggt acg atg ggt tga g-b (5′)	CPP Library Adaptor
Right adaptor	(5′) b-tgc acc taa tct tcc ata gct tca ctc gca gac att cta c-b (3′) (3′) OH-acg tgg att aga tgg tat cga agt gag cgt ctg taa gat g-b (5′)	CPP Library Adaptor
ClonalRCA primer I	(5′) Biotin-aaa aaa aat tcg acc tat cct tgc gca gct cga g-OH (3′)	Surface primer for Target 1 Clonal RCA
ClonalRCA primer II	(5′) Biotin-aaa aaa aac cat gaa caa aat gtg act cat atc-OH (3′)	Surface primer for ClonalRCA of Target 1 Circles
Spacer oligonucleotide A	(5′) Biotin-aaa aaa aa-OH (3′)	Surface primer for ClonalRCA of Target 1 Circles
ClonalRCA primer III	(5′) Biotin-ttt ttt ttt ttt ccc tgt aca ctc aac caa ct-OH (3′)	Surface primer for ClonalRCA of CPP Libray
ClonalRCA primer IV	(5′) Biotin-ttt ttt ttt ttt gtg aag cta tgg aag att ag-OH (3′)	Surface primer for ClonalRCA of CPP Libray
Spacer oligonucleotide B	(5′) Biotin-ttt ttt-OH (3′)	Surface primer for ClonalRCA of CPP Libray

(Merck, 1.02931) and incubated for 6 h at 50 °C. Residual amino groups are end-capped with succinic anhydride (Aldrich, 239690). After further washing steps with DMSO and PBS-T buffer (PBS supplemented by 0.05% Tween 20 and 0.515 g/l ProClin™ 300), the flow channels are incubated for 2 h with a solution of 1 mg/ml Neutravidin in PBS-T buffer. Finally, the flow cells are rinsed several times with PBS-T buffer and stored in a refrigerator at 4 °C until further use.

Immobilization of primers to the surface

Immobilization of biotinylated primers to the neutravidin-coated surface is achieved by incubating the streptavidin-coated surface (StreptaWell strips, Roche, Cat.No. 11664778001) or the neutravidin-coated surface (GeneRead flow cell; QIAGEN) and the mixture of biotin-forward primer (0.165 μM), biotin-reverse primer (0.165 μM), and biotin-spacer oligonucleotide (0.66 μM) in Immobilization Buffer [50 mM Tris–HCl (pH7.5),10 mM MgCl2, 1000 mM NaCl, 0.0012% Tween20, 0.15% NP40] for 1.5 h at room temperature. After immobilization, the surface was washed twice at 65 °C in Immobilization Buffer. All biotinylated primers were ordered from Integrated DNA Technologies (IDT). We used ClonalRCA Primer I, ClonalRCA Primer II and Spacer Oligonucleotide A for the immobilization for target-1 ClonalRCA. The oligonucleotides ClonalRCA Primer III, ClonalRCA Primer IV, and Spacer Oligonucleotide B were used for ClonalRCA of Circular Panel PCR Library DNA (CPP-library DNA). All oligonucleotides are listed in Table 1.

Hybridization of DNA to primers immobilized to the surface

Prior to hybridization, circular DNA (Target-1 circles. CPP-Circles) is denatured by incubating the circles for 5 min at 95 °C in 50 mM Tris–HCl (pH 7.5),15 mM $MgCl_2$, 120 mM NaCl, 0.0012% Tween20 followed by chilling on ice. The hybridization of circular target DNA is achieved by applying the denaturated circles (100 pg target-1 DNA or 1 ng Circular Panel PCR DNA) to the surface. Hybridization is performed for 15 min at room temperature. After the hybridization, the hybridization mixture is aspirated and the surface is washed at room temperature with 37.5 mM Tris–HCl (pH 7.5),10 mM $MgCl_2$, 50 mM KCl, 20 mM $(NH_4)_2SO_4$ to remove non hybridized circles from the surface.

ClonalRCA

The ClonalRCA reaction is started by adding a mixture of a ClonalRCA Buffer [37.5 mM Tris–HCl (pH 7.5),10 mM $MgCl_2$, 50 mM KCl, 20 mM $(NH_4)_2SO_4$,1.25 mM dATP, 1 mM dCTP, 1 mM dGTP, 1 mM dTTP] and REPLI-g Midi Polymerase (1 μl/80 μl reaction mixture). The reaction is incubated for 2 h at 38 °C and stopped by heating to 65 °C and aspiration of the reaction mixture. After ClonalRCA reaction, the reaction mixture is aspirated and the surface is washed two times at room temperature with 37.5 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM KCl, 20 mM $(NH_4)_2SO_4$ to remove non bound RCA products from the surface. After the washing step, the flow cell is stored in washing buffer until further use at 4 °C. Staining of clusters by YOYO-1 is performed in a Solution comprising 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, YOYO-1 Iodide (freshly added 1:30 000 diluted). After staining the solid surface is washed three times.

Determination of the amplification factor F

ClonalRCA clusters are eluted by adding 50 μl reconstituted DLB (REPLI-g Single Cell kit; QIAGEN, Cat.no. 150063), incubating for 1 h at 37 °C, followed by the addition of 50 μl Stop Solution (REPLI-g Single Cell kit). One microliter of the eluate is applied to real-time PCR amplification (Quantifast SYBR Green PCR kit, Cat.No. 204141) using the appropriate primers (Table 2). All primers are purchased from Integrated DNA Technologies (IDT). Thermocycling was performed on an ABI 7900 real-time cycler instrument for 5 min 95 °C (initial denaturation) and 45 cycles for 10 s, 95 °C and 30 s 60 °C followed by a melting curve analysis.

Table 2: Primers for determination of amplification factor

Name	Sequence	Circle size incl. adaptor sequence (nt)	GC content of target sequence (%)	PCR Amplicon size (nt)	Comment
Primer 80	ctg tgt atc aat gtc atg cc	101	45	102	Circular Target-1 qPCR; primers for inverted PCR span the ligation site
Primer 81	gtt aga cga tag gac agt aca				
DDR2-2 for	gga tga att act ttg taa tgg cta ctc	268	31	179	qPCR of DDR2-2 target; CPP-Library,
DDR2-2 rev	caa tgc cgg gta act ttg aa				
DDR2-3 for	gcc aga ttc cag atg agg ac	226	52	137	qPCR of DDR2-3 target; CPP-Library,
DDR2-3 rev	cca gaa cat ggg ctt tct tg				
KRAS for	tct ttt aat ttg ttc tct ggg aaa	248	31	159	qPCR of KRAS target; CPP-Library,
KRAS rev	cat tgt ttt ctt tca gcc aaa				
IDH2 for	gca gag aca aga gga tgg ct	248	64	159	qPCR of IDH2 target; CPP-Library,
IDH2 rev	aac atc cca cgc cta gtc c				

Table 3: Primers for primer extension experiments

Name	Sequence	Comment
Primer 811	agg cat gtg gtt aga cga tag gac agt aca	Forward primer extension
Primer 802	agg cat gtg ctg tgt atc aat gtc atg ccc atga	Reverse primer extension
Primer 810	agg cat gtg gtt aga cg	Primer 810 and Primer 80 used for quantitation of forward primer extension product
Primer 800	agg cat gtg ctg tgt atc	Primer 800 and Primer 81 used for quantitation of forward primer extension product

A total of 0.1–100 pg of the corresponding amplicon is used as a standard for DNA quantification. As a control we use the target DNA that is hybridized to the oligonucleotides immobilized to the surface but is not amplified by ClonalRCA. In other reaction without quantification standards, the amplification factor F is calculated by the formula $F = 1.95^{\mathrm{Delta}-Cq}$ corresponding to a PCR amplification efficiency of ~97%. The Delta $-$ C_q value is obtained by the C_q (DNA eluted from microwell after RCA) subtracted by the C_q (DNA eluted from microwell after target hybridization prior to RCA).

Primer extension

For primer extension experiments, we hybridize the appropriate primer (final concentration: 1 μM; for sequence, Table 3) in Primer Hybridization Buffer [50 mM Tris–HCl (pH 8.8), 100 mM NaCl, 15 mM $MgCl_2$; 55% PEG300] using a touch down temperature profile from 90 °C to 65 °C. After primer hybridization, the surface is washed to remove non-hybridized primer and the primer extension mix [20 mM Tris–HCl (pH 8.8), 50 mM KCl, 10 mM $MgCl_2$; 10 U Bst DNA Polymerase] is added and incubated for 10 min at 56 °C. After primer extension, the surface is washed again and eluted (see above) for real-time PCR analysis (see above) of ClonalRCA product and primer extension products.

Sequencing analysis

For sequencing ClonalRCA clusters, we use the standard GeneRead Sequencing Q kit (QIAGEN; cat.no. 185201), the GeneRead Sequencing Buffer Q kit (QIAGEN, cat.no. 185901) containing terminated dCTP-Alexa488, dUTP-R6G, dATP-ROX, and dGTP-Cy5 and the GeneReader instrument (QIAGEN, cat.no. 9002312). Sequencing runs are usually performed for 20 cycles under standard settings with the exception of manual z-axis adjustment. In contrast to classical sequencing runs with clusters immobilized on spherical beads, ClonalRCA clusters are generated directly onto the flat flow cell surface. Therefore, manual z-axis adjustment is necessary. Sequencing runs are not analyzed by the standard GeneReader software. Instead of the GeneReader software, we use the free image analysis software ImageJ (https://imagej.net/) for sequencing analysis of image data. For the definition of relevant spots the images of all four color channels from the first cycle are analyzed. The ImageJ function > Find maxima < is used with a manually adjusted threshold for each image. Overlapping spots are filtered out by comparison of coordinates. Images from the same field of view of all cycles and channels are combined in one stack and registered using an ImageJ plugin > Template Matching and Slice Alignment< (Qingzong Tseng). The local maxima and minima of the regions that are defined as spots are determined for each of the aligned images. Custom ImageJ macros are used to support this semi-automated analysis approach. The resulting data are used for further calculations (using Excel and Scripts written in Visual Basic for Applications). Briefly, in a first step the differences between the local maxima (Max_{local}) and the local minima (Min_{local}) are calculated for each single cluster. The resulting signal differences (S_D) are used for further analysis. For each channel, the mean value of all S_D-values was calculated (M_C). Based on the channel specific mean values a total mean value is determined (M_T). Additionally, an individual S_D-mean value is determined for each spot over all channels (M_S). The relative spot brightness (B_S) is calculated by dividing the M_S through the M_T. Assuming that the M_C is below an expected positive signal and above a false positive signal, a fraction of this value is subtracted from the S_D values of all spots of the corresponding channel. The fraction is determined by the multiplication of the M_C with the B_S and an empirically determined correction factor (CF) of 0.8. This is done to consider the differences in the signal intensities of the spots. The resulting values are multiplied with an empirically determined channel-dependent correction factor to consider the channel-specific differences in signal intensities. Base calling is done by comparing the resulting values of each channel for each

cycle and defining the base with the highest value as the correct one. The resulting formula is shown below [1].

$$Value = (S_D - 0.8 \times B_s \times M_C) * CF = ((Max_{local} - Min_{local}) - 0.8 \times M_s/M_T \times M_C) * CF \quad (1)$$

Results

ClonalRCA mechanism

The process is outlined in Fig. 1. Cluster generation by ClonalRCA started with the generation of a single-stranded circular DNA library (ssc DNA Library). The protocol included all steps well known from NGS library formation including DNA fragmentation, end repair of DNA fragments, and the ligation of adaptors. In addition to the standard NGS library process, fragments were circularized by ligase reaction followed by DNA denaturation to get single-stranded circular DNA of the ssc DNA library. Therefore, both strands [(+) strand and (–) strand] are present in ssc DNA library but bind independently to separate sites on the surface. Two primers were immobilized onto the surface. One primer (forward primer) was complementary to the adaptor region within ssc DNA (–) strand. The other primer (reverse primer) hybridized to the ssc DNA (+) strand. After hybridization of ssc DNA library, non-bound circles were eliminated by an excess of washing buffer followed by the addition of the ClonalRCA reaction mix. Cluster generation (DNA amplification) was carried out for 2 h on a thermocycler with an *in situ* block using the strand displacement polymerase of the Phi29 phage. Because forward and reverse primers were immobilized on the surface, both strands were amplified within a single cluster during the exponential ClonalRCA reaction on the solid surface. During amplification, the first strand extended from one of these primers (e.g. forward primer) forms a concatemer complementary to the ssc target molecule hybridized to the primer. This first strand concatemer folded back and hybridizes to the other primer (e.g. reverse primer) which in turn is elongated to form another concatemer complementary to the first strand product. It is very likely that the first strand concatemer hybridized to multiple other primers so that multiple reverse strand products are formed. Reverse strand products hybridized to its complementary primers immobilized on the surface so that new forward strand products are synthesized. Finally, a DNA cluster is generated on the surface comprising concatemers of (+) and (–) strands of the target circle.

Determination of the amplification factor and cluster size

The original RCA is carried out in solution. Here we used solid phase ClonalRCA to generate amplification products that expands radially on the planar surface and form spots of DNA concatemers folded back to the surface. In general, RCA processes are limited by the probability to form concatemer–primer hybrids. Several factors influence the re-hybridization of amplified DNA to primers bound to the surface. It has been reported that hybridization on planar surfaces is 20- to 40-fold reduced and is affected by secondary structures and sterical hindrance [14–16].

In order to determine the amplification factor, we used initially a circular DNA generated after ligation of a 101 nt long oligonucleotide (target1). ClonalRCA Primer I and II are immobilized stoichiometrically on a streptavidin-covered microwell. In order to achieve a less tight cluster compaction, we added a spacer oligonucleotide (Spacer Oligonucleotide A) during primer immobilization. After DNA target circle hybridization, non-hybridized DNA was eliminated by aspiration the hybridization solution and the use of an excess of wash buffer. One set of microwells was used for ClonalRCA using a strand displacement polymerase (Phi29 polymerase). In a control set of microwells, a primer extension reaction was performed using a DNA polymerase (T4 DNA polymerase) without strand displacement activity. After the reaction, the DNA was eluted from surface and is quantified by real-time PCR with a target-specific primer set (primer 80 and 81). For the circular starting template, we obtained a *Cq* value of ~24 after

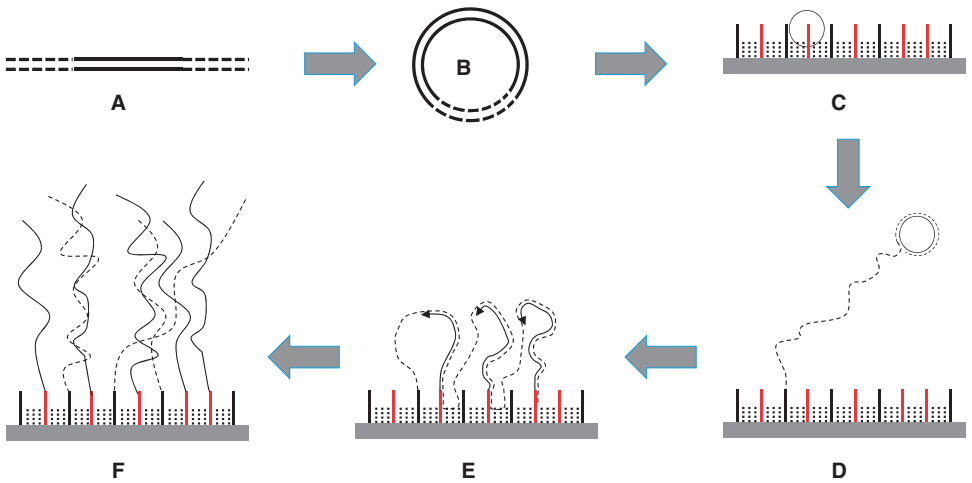

Figure 1: Illustration of the ClonalRCA mechanism. (**A**) Linear template molecules with left and right adaptor arms (dotted line) are used for (**B**) ligase reaction to form circular template molecules. (**C**) After denaturation of circular template DNA, DNA is hybridized to primers immobilized to the solid support (horizontal bar in gray). (+) DNA strand and (–) DNA strand binds to the primers because, forward (black vertical lines on surface) and reverse primer (red vertical lines on surface) are immobilized to the surface. In addition to forward and reverse primer, spacer oligonucleotides (dotted vertical lines on surface) are immobilized to the solid support. The spacer oligonucleotides are used to regulate the DNA copy number and the DNA crowding within the cluster. After hybridization all non-hybridized circles are eliminated by an intense washing step. Thereafter an amplification reaction mixture is added and the surface is incubated at 38 °C for 2 h. During this time, the first strand is synthesized from the target circle (**D**) which re-hybridized to the complementary primers immobilized on the solid support and primer extension occurs (**F**). During the reaction progress, less primers are available for re-hybridization. Therefore, more and more single-stranded DNA cannot re-hybridize and remains single-stranded (**F**).

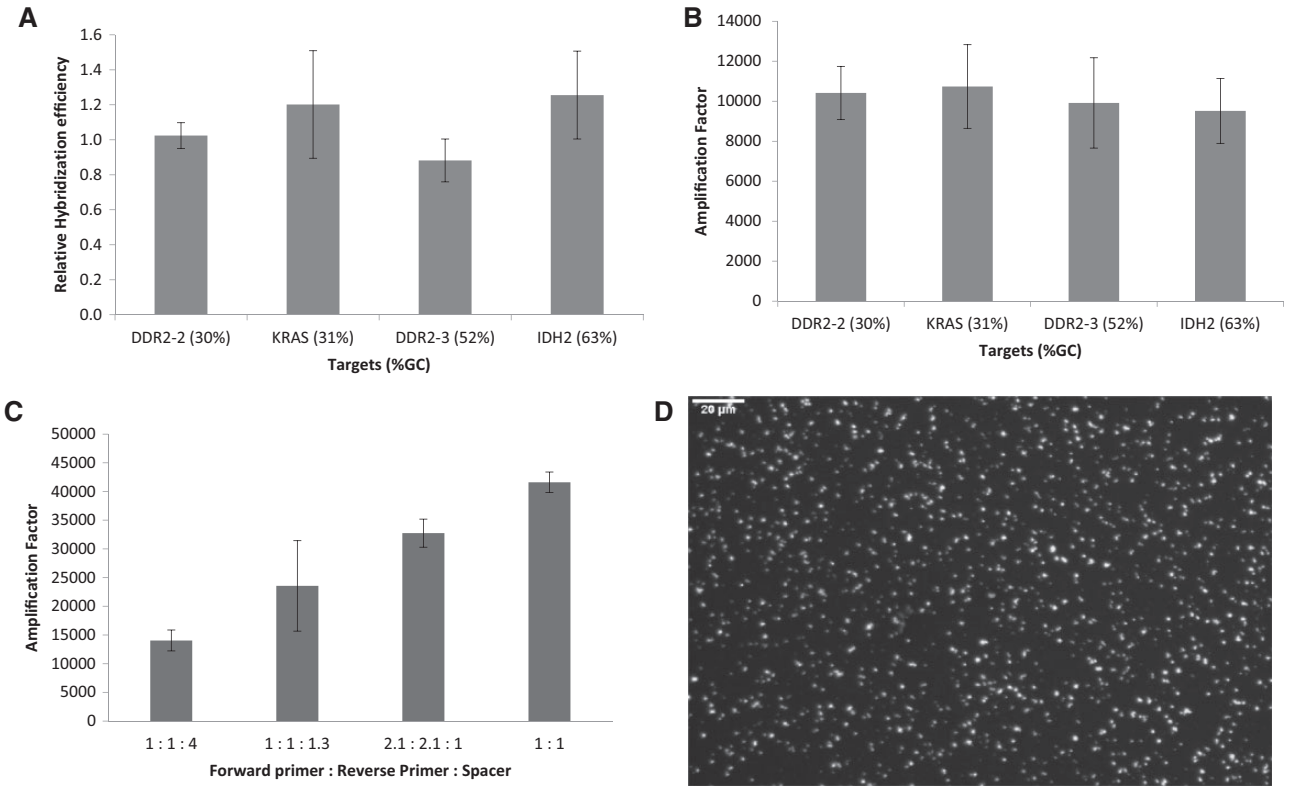

Figure 2: Demonstration of ClonalRCA. (**A**) In order to show the hybridization efficiency independent of GC content, a circular library from a PCR tumor panel was hybridized to the primers immobilized on a surface. A real-time PCR was performed from hybridized circles of 4 of the ~600 target circles (DDR-2, KRAS, DDR2-3, and IDH2). The four targets span approximately the GC content of the whole library. The relative hybridization efficiency was very similar and showed no significant difference between targets of different GC content ranging from 30% to > 60%. (**B**) The average DNA copy number of the ClonalRCA cluster (amplification factor) was determined after ClonalRCA by real-time PCR. ClonalRCA resulted in an amplification factor of ~10 000-fold in average independent of the GC content of the target circles. (**C**) The surface was coated with forward primer, reverse primer, and the short spacer oligonucleotide in a ration of 1:1:4. In order to increase DNA copy number per cluster and the DNA density within a spot, the fraction of spacer oligonucleotides bound to the surface was reduced to zero. The amplification factor increased from 13 000-fold to > 40 000-fold indicating that the ratio of the spacer oligonucleotide compared to the specific primers binding the target circles regulated the copy number per cluster. (**D**) ClonalRCA clusters were analyzed on a flow cell surface using YOYO-1 as DNA specific dye. In order to make sure that no monoclonal clusters are fused, a limited amount of DNA was used for hybridization. The spots were homogeneously distributed, were of a round shape and showed a size of ~1.2–2 µm.

hybridization and a C_q value of ~10 after RCA. The amplification factor F was calculated by the formula $F = 1.95^{\text{Delta}-C_q}$. The Delta$-C_q$ value was obtained by the C_q (DNA eluted from microwell after RCA) subtracted by the C_q (DNA eluted from microwell after target hybridization prior to RCA). We calculated an amplification factor of ~10 000 for the target-1 circular sequence (data not shown).

In order to determine the influence of GC content of target circles on hybridization and ClonalRCA, we determined amplification rates of various sequences from a circular PCR panel library (CPP-library). The PCR panel comprised exon fragments of 24 human clinically relevant tumor genes. The PCR panel was used for circular library formation and ClonalRCA on streptavidin-coated microwells. In these experiments, we determined C_q values for the hybridized DNA circles and for the RCA reactions. We determined the relative hybridization efficiency of four different targets (DDR2-2, DDR2-3, KRAS, IDH2) differing in the GC content from 30% to 63%. The relative hybridization efficiency was calculated from the C_q value normalized to the average C_q value of all targets. We found negligible differences in hybridization efficiency among the four targets (Fig. 2A). We further determined the amplification factor of all four targets as described above. The C_q values of RCA products generated on the surface (average: 13.3 ± 1.13) were 13.7 cycles lower than the C_q values obtained after circular library hybridization (27.1 ± 1.08). In real-time PCR experiments using dsDNA fragments as standards, we calculated an average amplification rate for all four targets of 10 150fold (±540) (Fig. 2B).

The amplification rate could be adjusted within a cluster by (i) changing the reaction time and (ii) the relative concentrations of specific primers and spacer oligonucleotide immobilized on the solid surface. In order to test the reaction time, we stopped the ClonalRCA (target-1 circle) after 1, 2, 3, or 4 h. Real-time PCR was used to estimate the quantity of ClonalRCA DNA. We detected a linear decrease of the C_q value from 12.0 (1 h), 10.9 (2 h), 10.0 (3 h), to 8.9 (4 h). This result indicated an ~8-fold increase of the average copy number per cluster within 3 h. The result was confirmed by analysing four representative sequences after ClonalRCA of the CPP-library (data not shown). In addition, we improved the amplification factor by increasing the concentrations of specific primers immobilized to the surface. In standard experiments, a relative concentration of forward primer and reverse primer and spacer oligonucleotide of 1:1:4 was bound to the surface for ClonalRCA. Reducing the fraction of the spacer oligonucleotide resulted in a ~3-fold higher amplification rate (Fig. 2C). In conclusion, the amplification rate can be adjusted to the specific needs of the downstream application.

In order to determine the DNA copy number/µm^2 on a surface, we performed the ClonalRCA reaction on a GeneReader

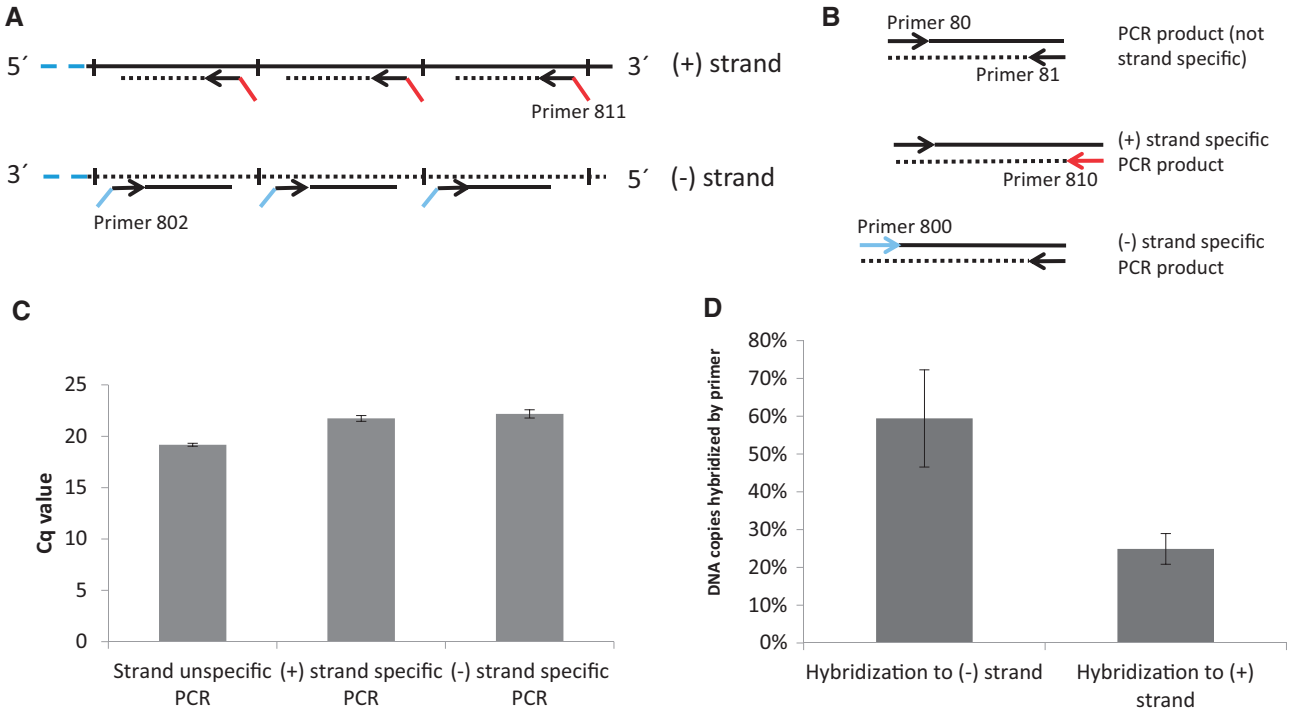

Figure 3: Presence of both strands after ClonalRCA (**A**) The (+) strand and the (–) strand were hybridized with strand specific primers tagged by different sequences (red sequence for reverse primer binding to the (+) strand and blue sequence for the forward primer). (**B**) After primer extension both extension products could be quantified individually during real-time PCR using the tags as primer binding site (while primer 810 bound to reverse primer extension product, primer 800 hybridized to forward primer extension product). In addition the total amount of ClonalRCA products and primer extension products could be determined by primers (Primer 80, 81) binding to the target circle sequence. (**C**) Both strand-specific real-time PCR reactions resulted in very similar *Cq* values indicating that both strands are present in ClonalRCA clusters. The *Cq* value of the primer pair binding to the target circle sequence was ~ 2.5–3 cycles lower than the *Cq* value of the strand specific real-time PCR reactions indicating a reduced primer hybridization efficiency. (**D**) Primer hybridization efficiency was determined in quantitative PCR experiments comparing strand-specific PCRs and strand-unspecific PCR. A primer binding efficiency of 22–28% [hybridization to (+) strand] and of 50–69% [hybridization to (–) strand] was determined.

flow cell and determined the average area of a cluster. The cluster were stained by using the DNA intercalating dye YOYO-1 iodide and imaged by the GeneReader instrument (Fig. 2D). In order to make sure that single spots were isolated and do not fuse, we hybridized a limited amount of circularized DNA (target1) to the flow cell. In this case, we analysed a single representative tile (one from 75 tiles) by Image J software and found ~25 044 spots covering 3.99% of the surface. The ClonalRCA clusters were homogeneously distributed. Although most spots were isolated, a minority of spots show an elongated shape and an increased size indicating fused clusters. Using the image analysis software Image J, we measured an average spot area of about 2.5 μm^2. As described above we calculated an amplification factor of ~10 000-fold. This means that every circle hybridized to the surface generated in average about 10 000 copies of DNA within a single cluster. This indicates that the DNA copy density per cluster in average is about 4000 copies/μm^2 under standard conditions.

Both DNA strands of the DNA template are generated within a single cluster

Keeping the ClonalRCA mechanism in mind, both DNA strands must be present within a single cluster. This means that ~10 000 copies of each of the strands were immobilized in a single cluster onto the surface. In order to determine the presence of (+) strand DNA and (–) strand DNA in ClonalRCA clusters, we used tagged primers for primer extension experiments. The tagged primers comprised a strand specific 3′-sequence and a 5′ tag. The 5′ tag sequence differed between (+) strand-specific primer and (–) strand-specific primer (Fig. 3A). In order to show that both strands were generated by the reaction mechanism, we performed ClonalRCA of single stranded target-1 DNA circle for this experiment. Based on target-1 circle sequence, we defined the (+) strand as the DNA sequence resembling the target circle and the (–) strand as the DNA sequence complementary to the hybridized target circle. After washing the surface, we hybridized tagged primers to ClonalRCA clusters and primer extension is carried out. We eluted primer extension products as well as the RCA clusters and analyzed them independently by real-time PCR with primers specifically hybridizing to the tag (Fig. 3B). As a control, we used a primer set amplifying both strands. In a typical experiment, we obtain very similar C_q values for (+) strand-specific primer and (–) strand-specific primer sets (in average 21.7 versus 22.2). In contrast, the primer set detecting both strand primer extension products (strand-unspecific PCR) and ClonalRCA DNA resulted in a C_q value 19.2 (Fig. 3C). This experiment clearly demonstrates that both strands are present within the ClonalRCA clusters. For the primer extension reaction, we used forward and reverse primers simultaneously. Therefore, a single primer extension product (forward or reverse) could be detected with one of the strand-specific PCR primer set. In contrast to strand-specific PCR, four strands could be quantified by the strand-unspecific PCR (detecting both primer extension products and both ClonalRCA DNA strands). Therefore, a C_q difference of about 2 was expected between strand specific PCR and strand-unspecific PCR. Because we found a C_q difference of 2.5 to 3 (21.7 or 22.2 versus 19.2), it was assumed that not all DNA copies are

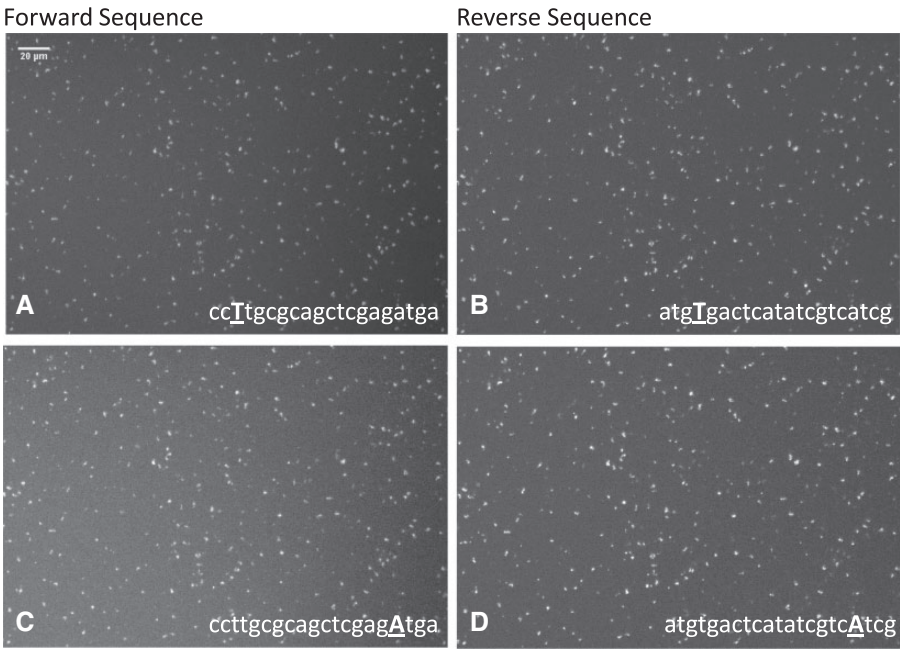

Figure 4: Forward and reverse sequencing of ClonalRCA clusters. Circular target-1 DNA was used for ClonalRCA. After forward sequencing the newly synthesized strand was removed by denaturation and reverse sequencing was initiated. Four representative images of the same position were shown with the relevant bases in bold letters: (**A**) Image of forward sequencing cycle 3 (base T, dUTP-R6G), (**B**) image of reverse sequencing cycle 4 (base T, dUTP-R6G), (**C**) image of forward sequencing cycle 17 (base A, dATP-ROX), (**D**) image of reverse sequencing cycle 18 (base A, dATP-ROX).

tagged by a primer. In order to determine primer hybridization efficiencies to the (+) strand and the (–) strand, we performed quantitative PCR using the tagged PCR products as standards. In doing so, we knew that the hybridization efficiency of (+) strand and (–) strand may differ because of imbalance of the copy number of both strands within a ClonalRCA cluster. Using the quantitative real-time PCR approach, we determined in average 2.4-fold more products generated by the (–) strand-specific primer hybridization (Fig. 3D) indicating a higher copy number of the first strand RCA product [or (–) strand product] compared to the second strand product [or (+) strand]. However, we cannot exclude a difference in hybridization efficiency of the strand specific tag primers. Comparing the quantity of the strand specific products and the RCA DNA copy number (Fig. 3D), we quantified that ~ 50–69% of the (–) strand product was hybridized to a primer while the (+) strand specific product was hybridized in 22–28% of strands indicating that not all RCA copies of the target sequence were hybridized to a strand-specific primer.

Both DNA strands of ClonalRCA clusters are accessible for sequencing reactions

The accessibility of forward and reverse strand was tested after ClonalRCA by a sequencing reaction. In order to get single clusters, we took 100 pg of target-1 for the experiment resulting in ~8.96 × 10^3 clusters/mm^2. After the ClonalRCA reaction, we hybridize the (–) strand specific primer and started sequencing for 20 cycles by the GeneReader chemistry without any adaption to surface bound cluster. Imaging of extended clusters was performed using the GeneReader instrument. In order to test both ends of target DNA, we denatured the first sequencing product and re-started sequencing using the reverse strand primer for 20 cycles (Fig. 4). For forward and reverse sequencing we detected an equal high number of mapping results. We mapped more than 90% of the clusters to the (–) strand and (+) strand. Only a minor number of clusters resulted in a sequence that could not be mapped to the target sequence. Most of them comprised an unusual nucleotide run [e.g. (C)n or (A)n]. Since the Phi29 DNA polymerase is not known as a polymerase generating such sequences, we interpreted these sequences by detection artefacts generated by dust or an non-optimal positioning of the flow cell during imaging (x, y, or z-axis). These results indicated that > 90% of all clusters comprised the right amplicon and both strands were present. In conclusion, both strands are accessible for downstream applications and can be sequenced one after another after ClonalRCA implicating the potential for paired-end sequencing.

Discussion

During the last decades, various methods have been developed to generate DNA clusters by amplification methods. Here, we describe ClonalRCA that combines the advantages of the proofreading Phi29 polymerase and an isothermal amplification driven by primers bound to the surface. The enzyme extend the primers immobilized on the surface while migrating along the DNA template strand, displacing the complementary strand, which in turn becomes a template itself for replication. Although Phi29 DNA polymerase results in a complete displacement of DNA strands, newly synthesized strands remain tethered within the cluster because all primers are immobilized on the surface. This reduces competing reactions and side products within the solution. In contrast to PCR-based amplification, ClonalRCA does not require different temperatures or other intermediate denaturation steps and ends in very long fragments with low mutation frequencies (10^{-5} to 10^{-6} errors per nucleotide; [17]). The strand displacing Phi29 DNA polymerase shows no tendency like Taq DNA Polymerase to delete short patches of DNA sequences by slippage [18, 19]. Slippage of polymerase without strand displacement activity (e.g. Taq polymerase) is highest in regions of strong hairpin

structures resulting in nonspecific amplification artifacts [19, 20] or loss of those regions. Therefore, we found a high amplification rate (~10 000-fold) independent of the tested target sequence.

The original RCA is carried out in solutions with amplification rates of up to 10^7-fold [21]. The much lower amplification rate of ClonalRCA is mainly caused by the surface bound amplification mode. The ClonalRCA process is limited by the probability to form concatemer–primer hybrids. Several factors influence the re-hybridization of amplified DNA to primers bound to the surface. It has been shown that DNA hybridization is affected if one of the hybridizing strands is bound to a surface. Gao found that hybridization of DNA to probes bound to a surface is 20- to 40-fold suppressed compared to DNA hybridization in solution. DNA hybridization is further decreased if the DNA forms secondary structures [16] and amplified DNA molecules will interact sterically [10]. Additionally, the amplification rate is limited by the two-dimensional radial DNA clusters growth. Radial cluster expansion results in an increased DNA density in the center of the cluster. As a consequence, the amplification rate is lowered in the centre of the cluster because DNA hybridization is sterically hindered and less primers are available for re-hybridization of amplified DNA strands [9, 10]. In contrast to PCR based solid-phase amplification reaction, ClonalRCA synthesize concatemers comprising multiple primer binding sites. Therefore, the probability of hybridizing of one of the primer binding sites to a primer immobilized to the surface is very high. All newly synthesized strands are tethered to the surface. Due to the strand-displacement mechanism, a DNA denaturation is not necessary for the amplification reaction.

The ClonalRCA mechanism results in the synthesis of (+) and (–) DNA strands. For further downstream analysis it is relevant whether single-stranded DNA or double-stranded DNA is the final product of ClonalRCA. During the RCA reaction long double-stranded DNA concatemers are generated at least as intermediate products. While the amplification reaction proceeds fewer primers are available and accessible in the centre of the cluster so that a displaced strand cannot be re-hybridized to primers immobilized on the surface. Therefore, one hypothesis is that the cluster comprises at least a fraction of single-stranded DNA at the end of the ClonalRCA reaction. Our results clearly demonstrates that both strands are present within the ClonalRCA clusters and can be applied for primer extension experiments as wells as for sequencing. In addition, the experiments show different amounts of forward and reverse primer extension products [~60% hybridization to (–) strand and 25% hybridization to (+) strand] which can be caused by various reasons. (i) Currently, we cannot exclude an imbalance of (+) strand and (–) strand copy number within ClonalRCA clusters. (ii) It may be that one of the primer binding sites is not accessible because the sequence is part of a hairpin structure. We can exclude this effect at least for experiments with target-1 DNA circles because target-1 DNA does not include hairpin structures at the relevant sites. (iii) In addition, we can exclude a primer hybridization hindrance because of a double-strand DNA that is formed by (+) strand and (–) strand. If both strands form a double-stranded DNA product, both primers would be affected similarly in primer extension reactions. Although the reasons remain unsolved, we found in preliminary experiments that the accessibility of DNA strands within a cluster can be affected. In primer extension experiments we measured a reduced primer hybridization and/or primer extension if clusters are formed on surfaces with a higher density of specific primers by reducing the fraction of spacer oligonucleotide proportion (data not shown). We assumed that the increased DNA density within the cluster resulted in reduced accessibility of the DNA within the cluster (Fig. 2C). However, it remains to be seen whether long sequencing reads of 100 nt and longer are possible using ClonalRCA clusters. The GeneReader settings today are not optimal for the sequencing of clusters immobilized directly on the flow cell surface. Today, the instrument is created for sequencing of clusters generated on beads. Therefore, the z-plane is adjusted to the beads and generates a different signal-to noise ratio. Although we approached the right z-plane, z-plane adjustment was not optimal. In addition, the standard software requires beads for image analysis and DNA clusters immobilized directly on to the surface cannot be analysed at a higher density.

The ability to generate clusters by an isothermal amplification process using a proofreading polymerase with strand displacement activity is highly attractive. The Phi29 DNA polymerase results in DNA with fewer errors and less slippage effects. As demonstrated in a variety of publications, the enzyme amplifies DNA with negligible sequence-dependent bias. ClonalRCA generates both DNA strands, (+) strand and (–) strand, in a single cluster. Both DNA strands are accessible for further downstream analysis without the need complicated manipulations.

In contrast to Bridge amplification where each amplification cycle requires the cyclic addition of denaturating reagents for strand separation, polymerase, and nucleotides, all steps are performed in a single ClonalRCA reaction utilizing the strand displacement Phi29 DNA polymerase. Therefore, ClonalRCA can be an efficient alternative amplification method to other methods forming DNA clusters on planar surfaces or beads.

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Conflict of interest statement. All authors are employees of QIAGEN GmbH, Germany.

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