View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Edinburgh Research Explorer

THE UNIVERSITY *of* EDINBURGH

Edinburgh Research Explorer

Ramified rolling circle amplification for synthesis of nucleosomal DNA sequences

Citation for published version:
van Emmerik, CL, Gachulincova, I, Lobbia, VR, Daniëls, MA, Heus, HA, Soufi, A, Nelissen, FHT & van Ingen, H 2020, 'Ramified rolling circle amplification for synthesis of nucleosomal DNA sequences', *Analytical Biochemistry*, vol. 588, 113469. https://doi.org/10.1016/j.ab.2019.113469

Digital Object Identifier (DOI):
10.1016/j.ab.2019.113469

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
Analytical Biochemistry

General rights
Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy
The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.

Download date: 11. May. 2020

Analytical Biochemistry 588 (2020) 113469

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Ramified rolling circle amplification for synthesis of nucleosomal DNA sequences☆

Clara L. van Emmerik[a], Ivana Gachulincova[b], Vincenzo R. Lobbia[a], Mark A. Daniëls[a], Hans A. Heus[c], Abdenour Soufi[b], Frank H.T. Nelissen[c], Hugo van Ingen[a,*]

[a] Bijvoet Center for Biomolecular Research, Utrecht University, the Netherlands
[b] MRC Centre for Regenerative Medicine, University of Edinburgh, United Kingdom
[c] Institute for Molecules and Materials, Radboud University Nijmegen, the Netherlands

ARTICLE INFO

Keywords:
Nucleosome
DNA synthesis
Ramified RCA
Transcription factor binding

ABSTRACT

Nucleosomes are a crucial platform for the recruitment and assembly of protein complexes that process the DNA. Mechanistic and structural *in vitro* studies typically rely on recombinant nucleosomes that are reconstituted using artificial, strong-positioning DNA sequences. To facilitate such studies on native, genomic nucleosomes, there is a need for methods to produce any desired DNA sequence in an efficient manner. The current methods either do not offer much flexibility in choice of sequence or are less efficient in yield and labor. Here, we show that ramified rolling circle amplification (RCA) can be used to produce milligram amounts of a genomic nucleosomal DNA fragment in a scalable, one-pot reaction overnight. The protocol is efficient and flexible in choice of DNA sequence. It yields 10-fold more product than PCR, and rivals production using plasmids. We demonstrate the approach by producing the genomic DNA from the human *LIN28B* locus and show that it forms functional nucleosomes capable of binding pioneer transcription factor Oct4.

1. Introduction

Nucleosomes are the repeating unit of chromatin, protecting genome integrity and regulating DNA-templated processes like transcription, replication and repair. This makes them a crucial scaffold for protein binding and therefore a highly attractive target for biochemical and structural studies. Nucleosomes are usually reconstituted *in vitro* from the individual histones, H2A, H2B, H3 and H4, and a ~150 bp DNA sequence, referred to as nucleosomal DNA. To obtain stable, homogeneous samples, the DNA sequence used, is typically a so-called strong-positioning sequences, such as the Widom 601 sequence [1] or the human α satellite repeat [2]. Although these DNA sequences are widely used in the field, there is a concern that the resulting nucleosomes are more stable than native ones and therefore do not accurately reflect the situation *in vivo*. Therefore, there is an increasing interest in the study of nucleosome dynamics and interactions reconstituted from alternative or genomic DNA sequences. Since structural studies by methods such as x-ray crystallography or NMR spectroscopy usually require milligram quantities of nucleosomes, there is a demand for efficient procedures to obtain nucleosomal DNA with any sequence of choice. We here present a tailored protocol to use rolling-circle-amplification to copy a template into a very long double-stranded repeat that upon cleavage results in the desired nucleosomal DNA fragment.

At present, two methods are regularly used to produce nucleosomal DNA in milligram quantities. Firstly, the DNA can be produced from a plasmid containing multiple repeats of the desired sequence [3]. The plasmid is transformed into *E. coli* and amplified by culturing the bacteria. The plasmid is then isolated and digested into the individual repeats, and the plasmid backbone is removed from the product sequence by ion exchange chromatography. In our lab, we get an average yield of ~20 mg of product from 3 L of culture using a 12-mer repeat of the 601 sequence; the Luger lab reported ~40–60 mg from 6 L of culture using a 24-mer repeat of the α satellite sequence. Alternatively, regular PCR amplification can be employed for nucleosomal DNA synthesis, starting from a plasmid containing a single copy of the desired sequence. This method is best used for small amounts, but can be scaled up, yielding 2–3 mg of pure product from 40 96-well plates in our lab. The plasmid method is usually preferred, as the yield is much higher. However, construction of the template plasmid can be challenging, forming a bottle neck when switching to different sequences. Although the single

Abbreviations: rRCA, ramified rolling circle amplification; PCR, polymerase chain reaction; dNTPs, deoxyribose nucleoside triphosphates
☆ Subject Category: DNA Recombinant techniques and nucleic acids.

* Corresponding author. Bijvoet Center for Biomolecular Research, Utrecht University.
E-mail address: h.vaningen@uu.nl (H. van Ingen).

https://doi.org/10.1016/j.ab.2019.113469
Received 15 August 2019; Received in revised form 7 October 2019; Accepted 7 October 2019
Available online 09 October 2019
0003-2697/ © 2019 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license
(http://creativecommons.org/licenses/BY-NC-ND/4.0/).

repeat plasmid used in the PCR method does allow sequence flexibility, the method is rather labor-intensive, and the yield is low.

A third option to produce DNA is based on rolling circle amplification (RCA). RCA requires a circular template and a polymerase that has a high processivity and strand displacement capacity, resulting in a long single-stranded product containing many repeats that are complementary to the template sequence. An advantage of RCA over PCR is that it is carried out in a one-step, isothermal reaction. RCA was originally developed for single-stranded amplification of so-called 'padlock' probes for specific DNA sequence detection [4–6]. Since then, RCA has been used in a wide variety of applications, ranging from single molecule detection methods to the synthesis of DNA nanostructures and materials (for a review see Ref. [7]). For large-scale synthesis of single-stranded DNA, the RCA protocol has been extended to include digestion of the long RCA product into monomers by 'cutter hairpins' [8] or by annealing of a complementary digestion splint to form double-stranded restriction sites [9]. This procedure has been used to produce 'monoclonal' single-stranded DNA oligonucleotides on a microgram scale [8] and single-stranded DNA aptamers on a multi-milligram scale [9].

RCA can be tuned to produce double-stranded DNA by the addition of a primer complementary to the product strand. This leads to ramification or (hyper)branching and an overall enhanced amplification factor of the reaction [10,11]. This technique, also known as cascade RCA of exponential RCA, has been exploited in diagnostic and biosensing assays as well as in sequencing of single cell genomes to improve detection of low abundance nucleic acid targets [10,12].

Here, we show that rRCA is an efficient and flexible method for large-scale production of dsDNA, in particular for nucleosomal DNA synthesis. The protocol introduced here enables the production of milligram amounts of double-stranded DNA in a scalable, one-pot overnight reaction. By proper design of the template sequence, the long double-stranded product can be directly digested into monomers by a dedicated restriction enzyme. We detail the procedure to design and construct the circular template, to efficiently digest the rRCA product and purify the desired nucleosomal DNA. Our procedure yields 2 mg from a 12 mL reaction, a 10-fold increase in yield compared to the same volume of PCR reactions. The major advantage of the method is the flexibility in choice of DNA sequence. We demonstrate the method by producing genomic DNA from the 3' end of the *LIN28B* locus and show that it forms functional nucleosomes capable of binding pioneer transcription factor Oct4.

2. Material and methods

2.1. Construction of the starting plasmid

The *LIN28B* template sequence, corresponding to the genomic location hg18-chr6: 105,638,004–105,638,165, was obtained as a sequence-verified gBlock® gene fragment (IDT) including a 15 bp extension on both sides that contain either *Eco*RI or *Xba*I restriction sites for cloning into pUC19 (Table 1). The ligated pUC19 vector containing the *LIN28B* gene fragment was used to transform *E. coli* (JM109) cells and the plasmid was purified and sequence-verified before further application.

2.2. Circular template synthesis

The pUC19 plasmid containing the *LIN28B* template sequence was amplified using standard PCR reactions with Pfu DNA polymerase (produced according to Ref. [13], construct available upon request). The template strand was purified by binding the biotinylated PCR product to Streptavidin Sepharose High Performance affinity resin (GE Healthcare), purification of the beads from the reaction mixture by repeated washing with Tris buffer (10 mM Tris HCl pH 7.5, 1 mM $MgCl_2$), and finally eluting the phosphorylated strand with 0.2 M NaOH. The eluent was neutralized by adding an equal volume of 0.2 M HCl and ethanol-precipitated. The template strand was circularized by heat-annealing the ligation splint and subsequently incubating this partial duplex with 200 U Taq DNA ligase (NEB) per 100 pmol of template in Taq DNA ligase buffer for 3 h at 45 °C. The circular template was then ethanol precipitated, reconstituted in T4 DNA ligase buffer and treated with 12.5 U Exonuclease I and 125 U Exonuclease III (Thermofisher) per 100 pmol of template at 37 °C for 1 h to remove the splint, unligated template and any other remaining single-stranded impurities. The circular templates were isopropanol precipitated and dissolved in deionized water to a concentration of 1 μM before use in ramified RCA.

2.3. Phi29 expression and assessment of activity

The gene for Phi29 DNA polymerase was amplified by PCR from a stock of Phi29 phages using primers 5′ACCATGGATCCCATATGCCGAGAAAGATGTATAG3′ and 5′ACCATGAATTCTCGAGTTATTTGATTGTGAATGTG3 (restriction sites underlined) and cloned into the *Nde*I and *Eco*RI sites of pET28a (Novagen) introducing a N-terminal His6-tag. This construct and detailed protocols for expression and purification are available upon request. Purification scheme is based on a previously described protocol for a His-GST-tagged version [14]. Briefly, the protein was expressed in either *E. coli* BL21 (DE3) cultivated in LB medium at 37 °C for 3 h, or in *E. coli* Rosetta2 cells (Novagen) in auto-induction medium (ZYM-5052) at 27.5 °C overnight [15]. After cell lysis by sonication in ice-cold buffer A (25 mM Tris-HCl (pH7.5), 0.5% Tween-20, 0.5% Nonidet P-40 substitute, 5% glycerol, 5 mM β-mercaptoethanol, 1 mM EDTA) with 20 μg/mL PMSF and 100 μg/mL lysozyme, the lysate was treated with DNaseI (2 U/mL) for 10 min at room temperature in presence 2.5 mM $MgCl_2$, 0.5 mM $CaCl_2$. The protein was purified from the supernatant by His-tag purification over a HisTrap HP Sepharose column (5 mL, GE Healthcare) pre-equilibrated in buffer A supplemented with 10 mM imidazole and 300 mM NaCl. Washing and elution were carried out with 25 and 300 mM imidazole, respectively. Polymerase containing fractions were pooled and subjected to another nuclease treatment with DNaseI (2 U/mL), exonuclease I (4 U/mL) and RNaseI (4 U/mL). Following a final His-tag purification, protein containing fractions were pooled and dialyzed overnight against 1 L of dialysis buffer (50 mM Tris-HCl (pH7.5), 100 mM NaCl, 1 mM DTT, 0.1 mM EDTA, 0.5% Tween-20, 0.5% Nonidet P-40 substitute and 50%

Table 1
DNA sequences of the *LIN28B* gene fragment and oligonucleotides.

Name	Sequence[a]
LIN28B insert	5'_ *GCATCGAATTCCC***G**G**G**GGTATTAACATATCCTCAGTGGTGAGTATTAACATGGAACTTACTCCAACAATACAGATGCTGAATAAATGTAGTCTAAGTGAAGGAAGAAGGAAAGGTGGGAGCTGCCATCACTCAGAATTGTCCAGCAGGGATTGTGCAAGCTTGTGAATAAAG**C**C**C***GGGTCTAGACTAGA*_3′
Starting primer	5′ GGGGGTATTAACATATCCTC 3′
Branching primer	5′ GGGCTTTATTCACAAGCTTGC 3′
Ligation splint	5′ TGTTAATACCCCCGGGCTTTATTCAC 3′

[a] Mutated residues from the original *LIN28B* sequence are indicated in bold, restriction sites for *Eco*RI and *Xba*I for cloning are underlined, extensions not part of the *LIN28B* sequence are in italic.

glycerol) at 4 °C. The Phi29 DNA polymerase isolate was aliquoted and stored at −20 °C. Activity of the isolate was determined by comparing product DNA yields relative to a commercial isolate (Epicentre).

2.4. Ramified RCA and digestion

The *LIN28B* circular template (20 nM) was amplified in a large-scale ramified RCA reaction in 1x CutSmart® buffer (NEB) with 200 U of Phi29 DNA polymerase and 0.5 U inorganic pyrophosphatase (NEB) per mL of reaction, 0.5 mM dNTPs, 2 μM of starting and branching primer and 4 mM DTT. Circular template and primer were heat-annealed in CutSmart buffer before adding dNTPs and the enzymes. The rRCA reaction was incubated at 30 °C for 5 h, followed by the addition of 1000 U *Sma*I (NEB) per mL of reaction and further incubation at 25 °C overnight. The resulting double stranded 162bp *LIN28B* DNA was purified by anion exchange chromatography on a HiTrap Q HP Sepharose column (5 mL, GE Healthcare) and ethanol precipitated.

2.5. Nucleosome reconstitution

rRCA-produced *LIN28B* DNA was mixed with human purified histone octamers at a 1:1.1 DNA:octamer molar ratio in the presence of 2 M NaCl and nucleosomes were reconstituted by salt gradient dialysis. The nucleosomes were analyzed on 6% TBE gel (Novex) in 1X TBE at 90 V for 1 h and visualized by Ethidium Bromide staining. The nucleosome concentration was calculated by quantifying the intensities of nucleosome bands, using free *LIN28B* DNA as the standard. The densitometric analysis of band intensities was performed using Multi-Gauge software (Fujifilm Science lab).

2.6. Mobility shift assay with Oct4

Full-length human Oct4 fused to an N-terminal 6X histidine tag with a thrombin cleavage site was expressed from a pET28b bacterial expression plasmid in *E. Coli* Rosetta (DE3) pLysS cells. The recombinant protein was purified under denaturing conditions over a His-SpinTrap column (GE Healthcare), desalted using a PD SpinTrap G-25 column (GE Healthcare) and concentrated using an Amicon Ultra-0.5 device (MW cut-off 10 kDa). EMSA was performed with increasing amounts of Oct4 to free *LIN28B*-DNA and *LIN28B*-nucleosomes. The free *LIN28B*-DNA (approx. 10 nM) and *LIN28B*-nucleosomes (approx. 25 nM) were incubated with recombinant Oct4 protein (12.5, 25, 50, 100, 200 nM) in DNA-binding buffer (10 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 10 μM $ZnCl_2$, 1 mM DTT, 10 mM KCl, 0.5 mg/ml BSA, 5% glycerol) at room temperature for 60 min. Free and Oct4 bound DNA were separated on 5% non-denaturing polyacrylamide gel run in 0.5X TBE at 90 V for 4 h. The gel was stained with ethidium bromide and visualized using SynGene G:Box.

3. Results

To illustrate the efficient production of any nucleosomal DNA sequences of choice by rRCA, we here focus on reconstituting nucleosomes using a sequence from a well-defined genomic locus in human fibroblasts. The *LIN28B* locus on chromosome 6 contains a well-positioned nucleosome that is the binding site for pioneer factors Oct4, Sox2, Klf4 and c-Myc (OSKM) [16] and is crucial in reprogramming and pluripotency [17,18]. A 162 bp sequence from the *LIN28B* locus was successfully used for OSKM binding assays by Soufi et al. [19], forming an excellent model system to test the functional quality of the nucleosomal DNA generated by ramified RCA.

The approach is outlined in Fig. 1. Briefly, a dsDNA sequence of choice is amplified from a storage vector, one of its strands is purified and circularized to yield the ssDNA template for RCA (Fig. 1A). In ramified RCA, this template is amplified using two primers, a starting primer and a branching primer, and Phi29 DNA polymerase. This enzyme is frequently used for RCA because of its high processivity and its strand displacement capacity [20], high 3′-5′ exonuclease activity and low error rate [21,22]. The starting primer anneals to the circular template and is elongated into a long, single-stranded repeat to which the branching primer can anneal. In regular RCA, only an equimolar amount of starting primer to circular template is needed, but in rRCA both starting and branching primer need to be in excess to template. Combined with strand displacement by Phi29, this results in the synthesis of a very long, branched, double-stranded repeat of the desired sequence. It should be noted that both starting and branching primer contribute to the ramification, as can be seen in Fig. 1B. Subsequent digestion and purification yields the final nucleosomal DNA fragment.

3.1. Design and storage of the template

The DNA sequence of choice will typically need minor modification to allow efficient use in rRCA. First, the template sequence needs to be designed to encode a restriction site for a blunt-end nuclease in the rRCA product such that the final nucleosomal DNA fragment can be released. In the most straightforward design, the restriction site is formed from the ends of the linear template, and thus overlaps with the ligation site (see Figs. 1 and 2). Therefore, accurate ligation of these ends into the circular template is essential as mutations in the restriction site impair digestion of the rRCA product and lower the final yield (see also below). Alternatively, the linear template can be designed in a way that the ligation site does not overlap with the restriction site, although care should be taken not to introduce ligation mistakes that interfere elsewhere, e.g. with nucleosome positioning or protein binding. If ligation is found to be inaccurate, the ligation site can be separated from the template sequence by encoding a digestion site on both the 3′- and the 5′-end of the desired sequence. Since the ligation site is then placed between the two restriction sites, it will be cut out of the rRCA product during digestion.

The choice of restriction enzyme can be guided by the sequence of the terminal bases in the desired nucleosomal DNA. As long as these bases are not part of a functional protein binding site, they can be altered at will since nucleosome positioning is encoded within the central 120 bp of the nucleosomal DNA. For *LIN28B*, we changed 4 residues at the termini of the original sequence to construct the recognition site for blunt-end cutter *Sma*I (CCCGGG), the first half of which (CCC) is at the 3′-end and the second half (GGG) at the 5′-end of the template sequence (Fig. 2A and B). Notably, we found that Phi29 is highly active in CutSmart® buffer when supplemented with 4 mM DTT. Thus, rRCA and digestion can be performed in the same reaction buffer, avoiding an additional buffer-exchange.

The RCA method starts from a circular, single-stranded DNA template. Single-stranded oligonucleotides up to 200 nucleotides are nowadays commercially available and could directly be circularized to use as an RCA template. However, the synthesis yield of such long oligonucleotides is usually limited to a few nanomoles and sequence inhomogeneity at the 5′-end can reduce ligation efficiency or result in incorrect ligation products. This not only lowers the amount of desired circular templates but may also give difficulties with the digestion of the product when the restriction site lies in or near the ligation junction. To create a robust stock of template, we therefore decided to generate the ssDNA template from a synthetic dsDNA gene. Such dsDNA fragments can be synthesized with highest sequence accuracy and can furthermore be stored in a vector, allowing for easy, reliable and low-cost amplification in *E. coli*. The desired ssDNA template can then be derived from this material as described below.

The *LIN28B* sequence was cloned in a pUC19 vector by extending the desired sequence with flanking restriction sites (Fig. 2C). Note that these extensions will not end up in the final nucleosomal DNA fragment. This sequence was ordered as a sequence-verified gBlock® double-stranded gene fragment (IDT). The final cloned constructs were

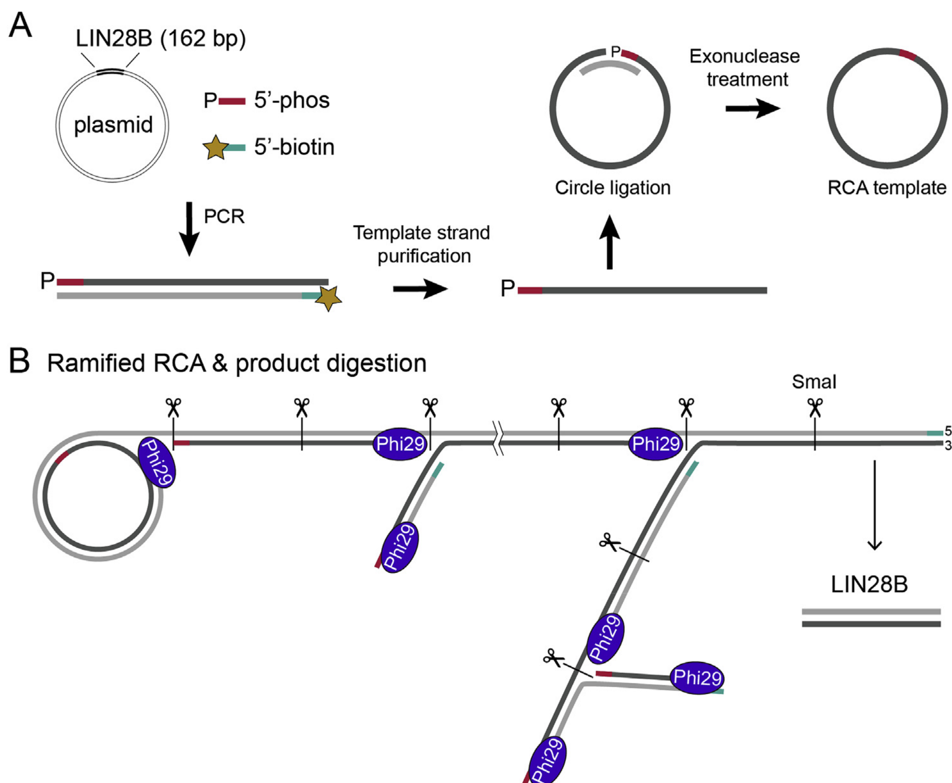

Fig. 1. Schematic workflow of ramified RCA for nucleosomal DNA synthesis. (A) Preparation of circular templates, starting from a plasmid with the desired nucleosomal DNA sequence, a 5′-phosphorylated primer and a 5′-biotinylated primer. The template strand (dark gray) is purified by removing the biotin-labeled strand (light gray) using streptavidin beads. The template strand is circularized using a splint to anneal the 5′- and 3′-end together for ligation. (B) Ramified RCA starting from the circular template, two primers (pink and teal), and Phi29 DNA polymerase produces a long, branched dsDNA product. Digestion is performed in the same reaction volume after addition of restriction enzyme *Sma*I (scissors) to release the double-stranded *LIN28B* product. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

verified by sequencing. This simple and straightforward cloning procedure can easily be performed with any other desired sequence and provides in a robust template for the preparation of rRCA single-stranded circles.

3.2. Preparation of the template circles

To obtain the ssDNA template from the storage plasmid, a PCR using two 5′-modified primers is required (Fig. 1A). The primer producing the template strand is 5′-phosphorylated, as the phosphate-group is necessary for ligation to the 3′-end. In order to purify this strand, the primer producing the other strand is 5′-biotinylated. Notably, either strand of a dsDNA molecule of interest can be chosen as template, as both will yield the same double-stranded final RCA product. By binding the PCR product to streptavidin beads and eluting the phosphorylated template strand with sodium hydroxide, pure single stranded product can be obtained. As the streptavidin beads have high binding capacity, no PCR product purification is necessary to remove excess biotinylated primers before binding to the streptavidin beads. This procedure yielded pure ssDNA *LIN28B* fragment in a straightforward manner (Fig. 3A).

To circularize the linear template strand a short ssDNA fragment, the ligation splint, is needed to anneal both ends together. This creates a double-stranded region with a nick that will be ligated using a DNA ligase. Although T4 DNA ligase is a very efficient and commonly used enzyme for ligating nicks, it is also capable of ligating the termini if there is a 1 or 2 nucleotide gap present or if the nick contains mismatches [23–25]. These gaps and mismatches could occur due to incorrect annealing of both ends on the ligation splint or by heterogeneity in the 5′-end of the primer used in PCR, commonly present in synthetic oligonucleotides. Since inaccurate ligation will cause mutations in the restriction site, special care needs to be taken to avoid this. We therefore used the thermostable Taq DNA ligase, which has a higher ligation accuracy and has no or little activity on gaps or mismatches at the ligation site [26]. Taking advantage of its thermostability, the accuracy of splint annealing can be further increased by carrying out the ligation at elevated temperature. For *LIN28B* a 26 nt ligation splint was used (see Table 1) in a ligation reaction with Taq DNA ligase at 45 °C, resulting in a homogenous pool of circular templates (Fig. 3B).

After ligation, the reaction mix not only contains the circular template but also left-over splint and linear template. These could prime either on the circle or on the product during RCA, resulting in single stranded by-products. To avoid these contaminations of the product, all linear ssDNA fragments remaining in the ligation are digested with exonuclease I and III. Fig. 3B shows this treatment results in pure circular templates. Sequencing analyses using the PCR primers confirmed that the purified circles contain the correct *LIN28B* sequence.

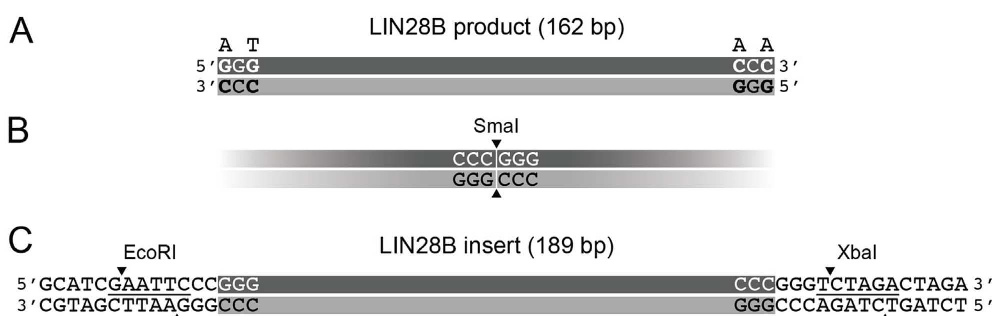

Fig. 2. Template design of the *LIN28B* sequence for use in ramified RCA. (A) Four bases of the original *LIN28B* sequence were mutated (in bold, original base indicated on top) to create a *Sma*I restriction site in the multimeric RCA product (B). (C) The extended *LIN28B* insert contains two restriction sites (*Eco*RI and *Xba*I, underlined) to enable cloning into the pUC19 vector.

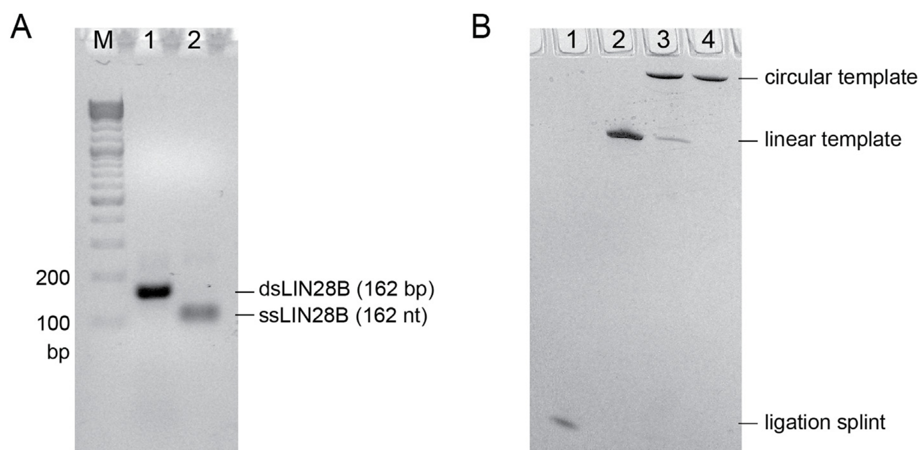

Fig. 3. Template strand production and circularization. (A) Agarose gel (2.5%, EtBr staining) showing template strand purification: crude PCR product (lane 1) and purified *LIN28B* strand (lane 2). (B) Denaturing PAGE gel (12%) showing circularization and exonuclease digestion of the template strand: Ligation splint (lane 1, 26 nt), purified template strand (lane 2, 162 nt), ligated template circles (162 nt), crude (lane 3) and after exonuclease clean-up (lane 4).

3.3. Optimization of ramified RCA reaction conditions

Ramified RCA is performed as a one-pot reaction at constant temperature (30 °C for Phi29 DNA polymerase). The optimal reaction buffer will depend on both the polymerase and restriction enzyme used, as mentioned above. For rRCA, the yield depends on the degree of branching and thus on the amount of starting and branching primer. Both primers need to be added in a 1:1 ratio to avoid contamination with ssDNA. To maximize the yield, also the amount of dNTPs and Phi29 DNA polymerase can be increased as these can become limiting. Finally, the purity of the circular template is essential to avoid by-products from remaining linear fragments (see above).

Initial rRCA reactions were performed using template circles that were purified after a standard 30-min exonuclease treatment. While the templates appeared pure on gel-analysis, considerable ssDNA *LIN28B* product (and multimers) were generated after digestion, indicating presence of residues linear template or annealing splint (Fig. 4A). By extending the exonuclease treatment three-fold, these by-products could be largely eliminated (Fig. 4A). Unexpectedly, in negative controls carried out without starting and branching primers, RCA product formation was still observed when starting from these pure template circles (Fig. 4B). This suggests that there are trace levels of DNA in the reaction mixture that can act as primer. These may originate from the Phi29 DNA polymerase stock, as co-purification of DNA is hard to avoid in preparations of this enzyme due to its high affinity for ssDNA and dsDNA [27]. Alternatively, trace levels of DNaseI in the Phi29 stock could generate linear ssDNA fragments capable of priming from the circular template directly.

To determine the optimal primer and Phi29 amounts, a series of 50 µL reactions were carried out using an excess of dNTPs (500 µM), and in presence of pyrophosphatase to avoid pyrophosphate-induced sequestering of Mg^{2+}. All reactions showed formation of a large RCA product that remains in the well of the agarose gel (see Fig. 4B and C, top panel). Since the product was difficult to pipet due to its high viscosity, the rRCA yield was assessed only after digestion by *Sma*I, resulting in *LIN28B* monomers of the expected size (162 bp) together with some dimers (324 bp) and multimers resulting from incomplete digestion (Fig. 4C, bottom panel).

First, the non-ramified control reaction, using only starting primer in equimolar ratio to the circular template, generated double-stranded product of the same size as *LIN28B* monomers, while a long single-stranded product was expected (Fig. 4C, lane 1). This may be caused by trace levels of DNA impurities as mentioned above, possibly in combination with aspecific priming of the starting primer or template-switching by Phi29 [28].

Second, addition of an equimolar amount of the branching primer showed double-stranded product formation with negligible increase in yield compared to the non-ramified reaction (Fig. 4C, lane 2). Under these equimolar conditions, the branching primer can anneal to the product as soon as a full ssDNA *LIN28B* repeat is synthesized, resulting in only one or a few repeats of double-stranded product and no ramification, leaving the majority still single-stranded.

Reactions with either 50- or 100-fold molar excess of both primers, showed significant and progressively increasing yield of double-

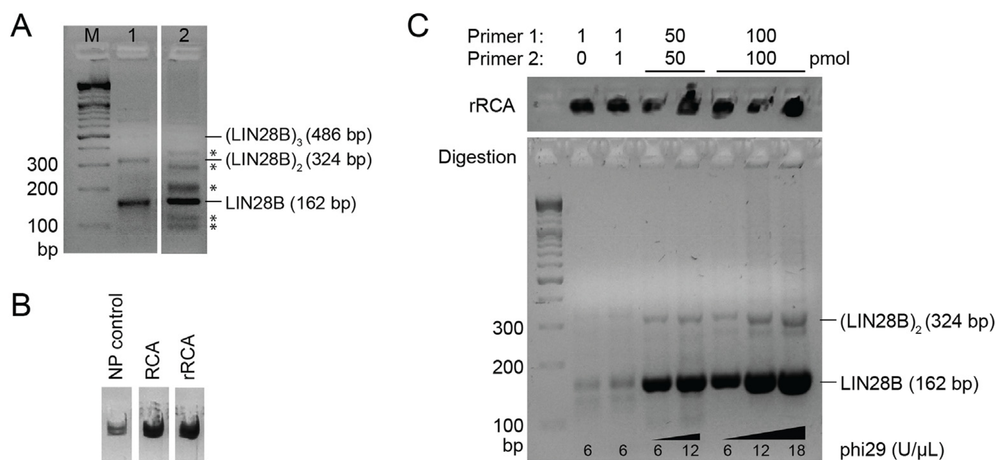

Fig. 4. Optimization of ramified RCA reaction conditions. (A) Agarose gel (2.5%, EtBr staining) showing digested rRCA products from a reaction with a clean circular template (lane 1) and with a circular template that still contained impurities (lane 2). Single-stranded by-products are indicated with an asterisk (*). (B) RCA products for 'no primer' (NP) control compared to product from RCA (1 pmol starting primer) and rRCA (50 pmol of both primers) on 0.8% agarose gel (EtBr staining). The circular template (1 pmol) used in this experiment was treated with exonuclease I and III for 1.5 h. (C) Optimization of primer amounts for ramification and adjustment of Phi29 DNA polymerase amount (2.5% agarose gel, EtBr staining). Top panel shows rRCA product, bottom panel shows *Sma*I-digested product. Before digestion, the rRCA yield is hard to compare, because of inconsistent pipetting due to high viscosity of the sample. Primer amounts are indicated above each lane, Phi29 concentrations are indicated below each lane.

stranded product, indicating successful ramification (Fig. 4C, lane 3 and 5). This confirms that effective branching only occurs at excess of both primers compared to the template. At these high primer concentrations, the amount of Phi29 DNA polymerase becomes limiting, as seen by the increase in yield upon adding two- or three-fold more Phi29 at constant primer concentration (Fig. 4C, lane 4, 6 and 7). Further increase of either primers or Phi29 may increase yield even more. Since very high excess polymerase is known to reduce polymerization efficiency, we choose to not use even higher excess of polymerase, but rather scale-up the reaction volume (see below).

In all cases, reaction time was limited to 5 h, at which point the reaction mixtures turned highly viscous due to product formation. We presumed reaction progress would be significantly reduced and thus little benefit would be gained by extending the reaction for longer.

While these results highlight a sensitivity of Phi29 DNA polymerase to the presence of trace levels of primers, large excess of starting and branching primer under ramified conditions will ensure correct priming and product formation. The results further demonstrate that maximum rRCA yield of dsDNA product is obtained at high molar excess of both primers in combination with elevated levels of Phi29 DNA polymerase. These conditions are most likely independent of the DNA sequence being produced.

3.4. Large-scale production, digestion & purification

For large-scale production of nucleosomal DNA, we take advantage of the fact that both rRCA and digestion are performed in a single reaction tube without the need of thermocycling. This means the reaction can easily be scaled up by increasing the reaction volume and reaction components accordingly. For large-scale production of *LIN28B* DNA, we performed both a 2 mL and a 12 mL synthesis in a single tube under conditions determined above for 5 h. In both cases, viscosity of the solution became very high at this point, which was quickly lowered after addition of the restriction enzyme *Sma*I. Digestion of the rRCA product into 162bp *LIN28B* monomers was almost complete after overnight incubation (Fig. 5A). The remaining larger fragments were separated from the main product by anion exchange chromatography using a very shallow salt gradient, resulting in pure nucleosomal DNA (Fig. 5B and C). We obtained 0.35 mg (3500 pmol) of purified product starting from 40 pmol of circular template in a 2 mL reaction volume, which is almost a 10-fold increase in yield compare to the same volume of regular PCR reactions. The 12 mL synthesis yielded 2.0 mg of pure *LIN28B*, demonstrating that the rRCA scales linearly with reaction volume.

3.5. Reconstitution of LIN28B nucleosomes and Oct4 binding

We next aimed to demonstrate that the nucleosomal DNA generated in our ramified RCA approach can be used to reconstitute functional nucleosomes. We used the *LIN28B* DNA from the large-scale production together with recombinantly expressed human histones to reconstitute human nucleosomes by salt-gradient dialysis, according to a previously published method [29]. The reconstitution was assessed by native PAGE analysis (Fig. 6A). A clear band shift was observed, indicating nucleosome formation. The efficiency of reconstitution was estimated from gel band intensities to be ~70%, which is comparable to what was previously published for *LIN28B* [19]. We thus conclude that rRCA derived nucleosomal DNA can be used for nucleosome reconstitution in the same manner as DNA from other sources.

To show that these nucleosomes are functional, we assayed their ability to bind pioneer transcription factor Oct4. Oct4 has been reported to associate with *LIN28B* nucleosomes *in vitro* in a sequence specific manner, as shown by DNase I footprinting [19]. Addition of recombinant Oct4 protein to our *LIN28B* nucleosomes induced a clear supershift in an EMSA experiment, indicating formation of an Oct4-nucleosome complex (Fig. 6B). Both the supershift and affinities for both free DNA and nucleosomes are in good agreement with previously published results [19]. Together, these results show that the rRCA-produced *LIN28B* DNA has the required quality in terms of purity and sequence to enable reconstitution of native nucleosomes capable of binding pioneer transcription factor Oct4.

4. Discussion

We here showed that ramified RCA is an efficient and flexible alternative for the large-scale production of nucleosomal DNA in milligram quantities. Compared to plasmid and PCR-based production, our rRCA protocol combines the flexibility in choice of sequence from PCR with the excellent yields of a multi-repeat product from the plasmid-based method. The straightforward design procedure requires only minimal changes to the terminal ends of DNA sequence of choice in order to create the circular template needed for RCA. A crucial aspect in the design of the template is the incorporation of a restriction site to digest the long, branched product. Judicious choice of restriction enzyme allows product digestion in the same reaction volume as the rRCA reaction, boosting the time-efficiency of the method. Production, digestion and purification can be performed in two days, the whole procedure including preparation of circular templates can be completed within a week. The yield is almost 10-fold higher than in the same volume of PCR reactions. Importantly, the one-pot rRCA reaction can easily be scaled up with a linear increase in yield. We here selected a

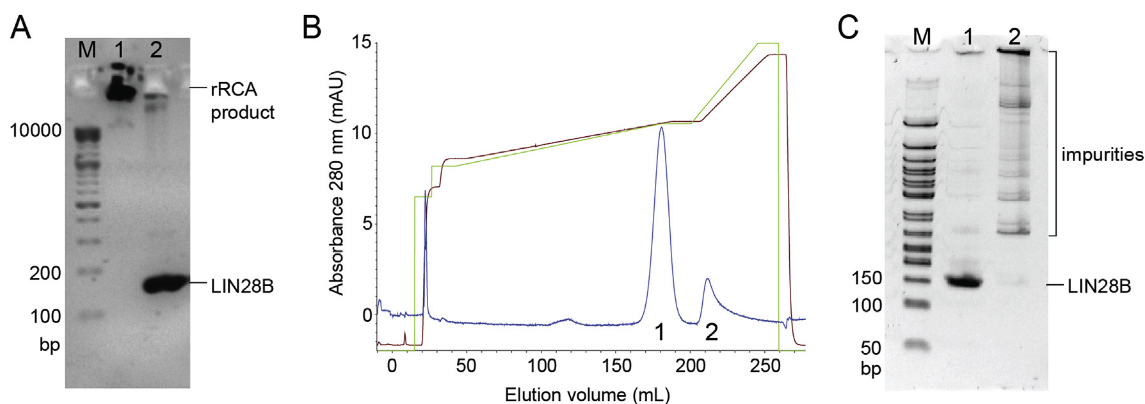

Fig. 5. Ramified RCA, *Sma*I digestion and ion exchange chromatography purification. (A) Ramified RCA product (lane 1) and *Sma*I digestion (lane 2) (B) Purification chromatogram of crude digested rRCA product using a 5 mL HiTrap Q HP column; A280 trace in blue, applied salt gradient in green (0–100% B, 1 M NaCl), measured conductivity in brown (mS). (C) 5% native PAGE gel showing the pooled fractions of peak 1 and 2, indicated in the chromatogram (panel B). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

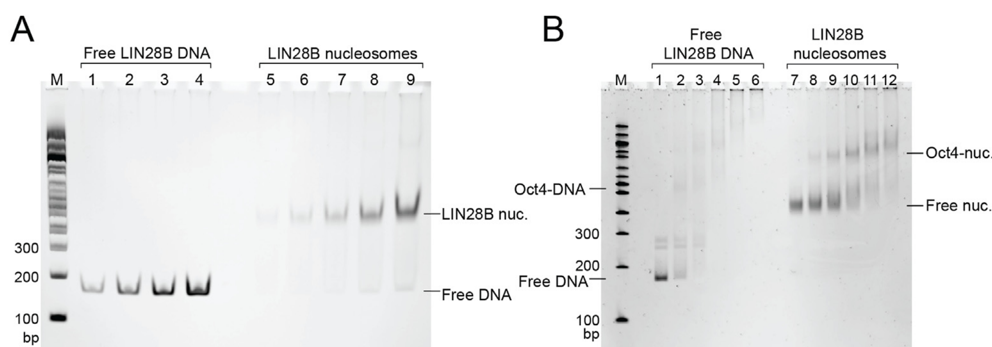

Fig. 6. (A) Nucleosome reconstitution with rRCA-produced *LIN28B* DNA (162bp) and human histones. Native PAGE (6%) lanes 1–4 contain 10, 25, 50 and 100 ng of free *LIN28B* DNA used for quantification of the nucleosome bands. DNA was mixed with histone octamer in a 1:1.1 ratio for reconstitution. Lanes 5–9 show increasing amounts of reconstituted nucleosomes estimated to contain 7, 17, 35, 71 or 142 ng of bound DNA, respectively, which is ~70% of the input free DNA. DNA in the nucleosome has approximately a 2-fold lower signal intensity compared to free DNA. (B) EMSA of Oct4 binding to free *LIN28B* DNA (10 nM) and assembled *LIN28B* nucleosomes (25 nM). Native PAGE (5%) lanes 1–6 and lanes 7–12 contain 0, 12.5, 25, 50, 100 and 200 nM of recombinant Oct4.

DNA sequence corresponding to a well-positioned nucleosome at the 3' end of the *LIN28B* locus. Since this sequence with 56.8% AT content has no particular features, we expect that the method can generally be applied to most DNA sequences, including larger templates for reconstitution of nucleosomal arrays on genomic DNA. Performance with highly repetitive sequences may be compromised by fidelity problems of the polymerases.

RCA requires a polymerase with extreme processivity and strand displacement, such as provided by Phi29 DNA polymerase. Since large-scale production runs also require high amounts of enzyme, in-house production is advisable to reduce costs (the construct is available upon request). Although this might be a barrier for labs that are not in need of continuous production of nucleosomal DNA, production is efficient (a 1 L culture provides Phi29 for approximately 4000 mL of rRCA) and the resulting Phi29 can be stored long-term at −20 °C.

Addition of pyrophosphatase was necessary to avoid the scavenging of Mg^{2+} by the formed pyrophosphate. This may be circumvented by performing the reaction in a dialysis bag in reaction buffer to avoid accumulation of pyrophosphate. Such a setup may improve yield by increasing buffer capacity and avoiding increases in phosphate concentration during the reaction, which could inhibit Phi29. The high viscosity observed towards the end of the rRCA reaction likely limits the diffusion of polymerase and reactants through the solution, and may thus reduce the potential yield. Performing rRCA and product digestion simultaneously, preventing the formation of very long *LIN28B* repeats, could therefore further improve the yield. However, this requires methylation of the circular templates in combination with a methylation-sensitive restriction enzyme, to avoid digestion of the template.

The rRCA yield was improved by increasing the primer to template ratio, as well as the dNTP and Phi29 concentration. Although rRCA can provide exponential amplification of the template as demonstrated at small-scale for Bst DNA polymerase [30], we did not observe this in our work. Analysis of the yield showed that the starting template was amplified effectively ~100-fold. The amplification here is presumably limited by the high concentration of the reactants and product leading to high viscosity. Dilution of the reaction could alleviate this, but lowered reaction rates may counteract reaching a higher yield. While further improvement in yield may be possible, we demonstrated here that our current protocol is effective and can furthermore be easily scaled. In comparison, exponential amplification in PCR is also only reached at very low template levels, thus requiring a large volume of reactions to reach milligram scale of purified product. In contrast, our rRCA protocol offers a highly practical execution without the need to prepare dozens of PCR-plates, and in particular when access to multiple PCR machines is limited. Moreover, the lack of thermocycling in rRCA leads to more efficient dNTP incorporation as a result of decreased degradation [9]. Even if our protocol relies on PCR to generate the template strand, one PCR plate will generate sufficient template for a ~36 mL RCA reaction, which would result in ~6 mg of DNA, equivalent to ~120 plates of PCR.

In conclusion, we presented a rRCA-based method to allow the efficient and flexible large-scale synthesis of nucleosomal DNA sequences, or any other dsDNA of comparable length. The protocol was used to produce multi-milligrams of human, genomic nucleosomal DNA with high purity. We demonstrated that rRCA-produced *LIN28B* DNA can be used to reconstitute stable and functional nucleosomes that are capable of binding pioneer transcription factor Oct4. Due to its ease of use and flexibility in sequence design, we believe this method is an ideal tool to produce a wide variety of nucleosomal DNA sequences to study the structure, dynamics, and interactions of nucleosomes or nucleosomal arrays, in particular of genomic nucleosomes as they occur *in vivo*.

Funding

This work was supported by a VIDI grant from the Dutch Science Foundation NWO to HvI (NWO-CW VIDI 723.013.010). AS was supported by an MRC career development award (MR/N024028/1). IG was supported by the BBSRC-EASBIO Ph.D training program (BB/M010996/1).

Declaration of competing interest

The authors declare no conflict of interest.

Acknowledgement

We thank dr. Gert Folkers for stimulating discussions and helpful suggestions and Yuri van Ekelenburg for purification of Pfu DNA polymerase.

References

[1] P.T. Lowary, J. Widom, New DNA sequence rules for high affinity binding to histone octamer and sequence-directed nucleosome positioning, J. Mol. Biol. 276 (1998) 19–42.
[2] K. Luger, A.W. Mader, R.K. Richmond, D.F. Sargent, T.J. Richmond, Crystal structure of the nucleosome core particle at 2.8 A resolution, Nature 389 (1997) 251–260.
[3] P.N. Dyer, R.S. Edayathumangalam, C.L. White, Y. Bao, S. Chakravarthy, U.M. Muthurajan, K. Luger, Reconstitution of nucleosome core particles from recombinant histones and DNA, Methods Enzymol. 375 (2004) 23–44.
[4] A. Fire, S.Q. Xu, Rolling replication of short DNA circles, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 4641–4645.
[5] D. Liu, S.L. Daubendiek, M.A. Zillman, K. Ryan, E. Kool, Rolling Circle DNA Synthesis: Small Circular Oligonucleotides as Efficient Templates for DNA Polymerases, (1996).
[6] J. Baner, M. Nilsson, M. Mendel-Hartvig, U. Landegren, Signal amplification of padlock probes by rolling circle replication, Nucleic Acids Res. 26 (1998) 5073–5078.
[7] M.M. Ali, F. Li, Z. Zhang, K. Zhang, D.-K. Kang, J.A. Ankrum, X.C. Le, W. Zhao, Rolling circle amplification: a versatile tool for chemical biology, materials science and medicine, Chem. Soc. Rev. 43 (2014) 3324–3341.
[8] C. Ducani, C. Kaul, M. Moche, W.M. Shih, B. Högberg, Enzymatic production of 'monoclonal stoichiometric' single-stranded DNA oligonucleotides, Nat. Methods 10 (2013) 647.

[9] F.H.T. Nelissen, E.P.M. Goossens, M. Tessari, H.A. Heus, Enzymatic preparation of multimilligram amounts of pure single-stranded DNA samples for material and analytical sciences, Anal. Biochem. 475 (2015) 68–73.
[10] P.M. Lizardi, X. Huang, Z. Zhu, P. Bray-Ward, D.C. Thomas, D.C. Ward, Mutation detection and single-molecule counting using isothermal rolling-circle amplification, Nat. Genet. 19 (1998) 225–232.
[11] D.Y. Zhang, M. Brandwein, T. Hsuih, H.B. Li, Ramification amplification: a novel isothermal DNA amplification method, Mol. Diagn. 6 (2001) 141–150.
[12] K. Zhang, A.C. Martiny, N.B. Reppas, K.W. Barry, J. Malek, S.W. Chisholm, G.M. Church, Sequencing genomes from single cells by polymerase cloning, Nat. Biotechnol. 24 (2006) 680–686.
[13] F.H.T. Nelissen, F.C. Girard, M. Tessari, H.A. Heus, S.S. Wijmenga, Preparation of selective and segmentally labeled single-stranded DNA for NMR by self-primed PCR and asymmetrical endonuclease double digestion, Nucleic Acids Res. 37 (2009) e114-e114.
[14] P.C. Blainey, S.R. Quake, Digital MDA for enumeration of total nucleic acid contamination, Nucleic Acids Res. 39 (2010) e19-e19.
[15] F.W. Studier, Stable expression clones and auto-induction for protein production in E. coli, in: Y.W. Chen (Ed.), Structural Genomics: General Applications, Humana Press, Totowa, NJ, 2014, pp. 17–32.
[16] A. Soufi, G. Donahue, Kenneth S. Zaret, Facilitators and impediments of the pluripotency reprogramming factors' initial engagement with the genome, Cell 151 (2012) 994–1004.
[17] J. Yu, M.A. Vodyanik, K. Smuga-Otto, J. Antosiewicz-Bourget, J.L. Frane, S. Tian, J. Nie, G.A. Jonsdottir, V. Ruotti, R. Stewart, I.I. Slukvin, J.A. Thomson, Induced pluripotent stem cell lines derived from human somatic cells, Science 318 (2007) 1917.
[18] N. Shyh-Chang, H. Zhu, T. Yvanka de Soysa, G. Shinoda, Marc T. Seligson, Kaloyan M. Tsanov, L. Nguyen, John M. Asara, Lewis C. Cantley, George Q. Daley, Lin28 enhances tissue repair by reprogramming cellular metabolism, Cell 155 (2013) 778–792.
[19] A. Soufi, M.F. Garcia, A. Jaroszewicz, N. Osman, M. Pellegrini, K.S. Zaret, Pioneer transcription factors target partial DNA motifs on nucleosomes to initiate reprogramming, Cell 161 (2015) 555–568.
[20] L. Blanco, A. Bernad, J.M. Lazaro, G. Martin, C. Garmendia, M. Salas, Highly efficient DNA synthesis by the phage phi 29 DNA polymerase. Symmetrical mode of DNA replication, J. Biol. Chem. 264 (1989) 8935–8940.
[21] J.A. Esteban, M. Salas, L. Blanco, Fidelity of phi 29 DNA polymerase. Comparison between protein-primed initiation and DNA polymerization, J. Biol. Chem. 268 (1993) 2719–2726.
[22] C. Garmendia, A. Bernad, J.A. Esteban, L. Blanco, M. Salas, The bacteriophage phi 29 DNA polymerase, a proofreading enzyme, J. Biol. Chem. 267 (1992) 2594–2599.
[23] S.V. Nilsson, G. Magnusson, Sealing of gaps in duplex DNA by T4 DNA ligase, Nucleic Acids Res. 10 (1982) 1425–1437.
[24] C. Goffin, V. Bailly, W.G. Verly, Nicks 3' or 5' to AP sites or to mispaired bases, and one-nucleotide gaps can be sealed by T4 DNA ligase, Nucleic Acids Res. 15 (1987) 8755–8771.
[25] D.Y. Wu, R.B. Wallace, Specificity of the nick-closing activity of bacteriophage T4 DNA ligase, Gene 76 (1989) 245–254.
[26] F. Barany, Genetic disease detection and DNA amplification using cloned thermostable ligase, Proc. Natl. Acad. Sci. U. S. A. 88 (1991) 189–193.
[27] H. Takahashi, H. Yamazaki, S. Akanuma, H. Kanahara, T. Saito, T. Chimuro, T. Kobayashi, T. Ohtani, K. Yamamoto, S. Sugiyama, T. Kobori, Preparation of Phi29 DNA polymerase free of amplifiable DNA using ethidium monoazide, an ultraviolet-free light-emitting diode lamp and trehalose, PLoS One 9 (2014) e82624.
[28] C. Ducani, G. Bernardinelli, B. Högberg, Rolling circle replication requires single-stranded DNA binding protein to avoid termination and production of double-stranded DNA, Nucleic Acids Res. 42 (2014) 10596–10604.
[29] Y. Tanaka, M. Tawaramoto-Sasanuma, S. Kawaguchi, T. Ohta, K. Yoda, H. Kurumizaka, S. Yokoyama, Expression and purification of recombinant human histones, Methods 33 (2004) 3–11.
[30] T.P. Beals, J.H. Smith, R.M. Nietupski, D.J. Lane, A mechanism for ramified rolling circle amplification, BMC Mol. Biol. 11 (2010) 94.