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Control of DNA replication *in vitro* using a reversible replication barrier

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Abstract

A major obstacle to studying DNA replication is that it involves asynchronous and highly delocalized events. A reversible replication barrier overcomes this limitation and allows replication fork movement to be synchronized and localized, facilitating the study of replication fork function and replication coupled repair. Here, we provide details on establishing a reversible replication barrier *in vitro* and using it to monitor different aspects of DNA replication. DNA template containing an array of lac operator (*lacO*) sequences is first bound to purified *lac* repressor (LacR). These substrates are then replicated *in vitro* using a biochemical replication system, which results in replication forks stalled on either side of the LacR array regardless of when or where they arise. Once replication forks are synchronized at the barrier, IPTG can be added to disrupt LacR binding so that replication forks synchronously resume synthesis. We describe how this approach can be employed to control replication fork elongation, termination, stalling, and uncoupling, as well as assays that can be used to monitor these processes. We also explain how this approach can be adapted to control whether replication forks encounter a DNA lesion on the leading or lagging strand template and whether a converging fork is present. The required reagents can be prepared in 1–2 weeks and experiments using this approach are typically performed over 1–3 days. The main requirements for utilizing the LacR replication barrier are basic biochemical expertise and access to an *in vitro* system to study DNA replication. Investigators should also be trained in working with radioactive materials.

INTRODUCTION

DNA replication and replication-coupled DNA repair are fundamental processes that suppress tumor formation and are targeted by chemotherapy[1]. A major obstacle to studying DNA replication is that it involves asynchronous and highly delocalized events[2–5]. This

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AUTHOR CONTRIBUTIONS

JMD developed the LacR barrier approach and related assays. JMD and TK adopted the LacR barrier approach to induce site specific uncoupling and developed related assays. JMD and EJV optimized and refined the LacR barrier approach to induce site specific uncoupling and related assays. JMD, SND, and MTC developed the approach to introduce site specific base modifications into template plasmid. EJV, TK, and SND performed the experiments shown. JMD and EJV wrote the manuscript with input from SND and TK.

Competing interests

No, I declare the authors have no competing interests as defined by Nature Research

variability has two main sources. First, within any given biochemical or cellular setup the establishment of individual replication forks is dispersed over many minutes to several hours[6–9]. Second, the rate of DNA synthesis at individual replication forks is highly stochastic[10,11]. This variability makes it challenging to perform time course analyses of DNA synthesis or analyze the replication fork structures formed during replication. Furthermore, the problem is compounded in metazoan systems where assembly of the replication forks is not restricted to specific sequences[12]. To overcome these limitations, a reversible replication barrier was previously developed[13]. This approach allows replication forks to be stalled at a specific region of template DNA and then restarted so that synchronous, localized DNA replication fork movement can be studied.

The reversible replication barrier approach is simple: an array of *lac* operator (*lacO*) sequences ('*lacO* array') is engineered into the template DNA sequence and bound to purified *lac* repressor (LacR) (Fig 1A) to create a barrier ('LacR barrier'). Replication of these substrates *in vitro* using a biochemical replication system leads to stalling of the replication forks on either side of the LacR barrier regardless of when or where they arise (Fig 1A). Once replication forks are synchronized at the barrier, IPTG is added to disrupt LacR binding so that the replication forks synchronously resume synthesis (Fig 1A). Importantly, both leading and lagging DNA strands resume synthesis[13], and the replication machinery exhibits the same behavior as when replication is monitored without a LacR barrier[13,14]. Thus, use of the LacR barrier does not appear to impact replication fork function.

The main advantages of the LacR barrier are: (1) it offers unparalleled synchronization and localization of DNA replication events, and (2) it allows for direct control of replication fork movement. Synchronization of DNA replication is a powerful tool to understand complex biochemical processes by ordering the events involved without the need remove or inhibit individual proteins. Localization of DNA replication allows for the detailed analysis of the DNA structures and nascent DNA strands during DNA replication. Control of replication fork movement allows replication fork encounters with an obstacle (e.g., a DNA lesion) to be analyzed in a strand-specific manner in the presence or absence of an opposing fork. In this protocol, we describe how to apply LacR barriers to study DNA replication and replication-coupled DNA repair and the assays that are typically used. Abbreviations used in this protocol are listed in Table 1.

Development of the protocol

The LacR barrier approach was initially developed to study the convergence of two replication forks during termination (Fig 1A). Large arrays of the bacterial transcription factors *lac* and *tet* repressor were reported to reversibly block replication in bacteria[15,16] but it was unclear whether the same would be true for eukaryotic systems and whether small enough arrays could be used to study replication on plasmid DNA templates. It was ultimately found that LacR was able to effectively and reversibly block DNA replication *in vitro* in *Xenopus* egg extracts (Fig 1A)[13] while *tet* repressor had essentially no effect on DNA replication[17], for reasons that remain unclear. The LacR barrier approach was initially used to elucidate a biochemical model for the termination of DNA replication[13] and to test

the role of fork convergence during the repair of a DNA interstrand cross-link[18]. It was quickly recognized that the LacR barrier was a robust approach to block DNA replication and could easily be introduced into a variety of plasmid DNA substrates. Thus, LacR barriers were engineered adjacent to DNA damage to investigate the mechanisms that take place when only a single replication fork encounters DNA damage[18–20]. This was achieved either by enzymatic modification of the DNA adjacent to the *lacO* array[17], or ligation of chemically-modified single-stranded[19] or double-stranded[18,20] DNA adjacent to the *lacO array*. LacR barriers have since been used to study replication fork stalling[21,22] and the normal functioning of replication forks[23]. Recently, we used the LacR barrier to induce unwinding of the replicative helicase in the absence of DNA synthesis ('uncoupling'), which we showed causes degradation of nascent DNA strands and replication fork reversal[24]. Thus, the uses of the LacR barrier have expanded significantly since its inception nearly a decade ago.

Applications of the method

This first step of the protocol is always to replicate plasmid DNA containing a LacR barrier *in vitro*. Once forks encounter the barrier, they stall, which permits the study of replication fork stalling itself (Figs 1B, 2A, 2B lanes 1–3,7)[21,22,25]. Disruption of the barrier by addition of IPTG causes replication forks to restart and complete DNA synthesis[13], as shown in Figure 2B (compare lanes 1–2 with 3–4). Doing so allows for the events that occur when two replication forks encounter each other ('termination') to be monitored (Figs 1C, 2A, 2B lanes 3–4)[13,14,26,27]. Approximately twelve LacR complexes bound to DNA (within a 365 bp *lacO* array) is sufficient to reversibly stall replication forks for several minutes[13]. The largest LacR barrier used to date binds 48 LacR complexes (within a 1490 bp *lacO array)*, which can stall replication forks for several hours[13,18,19]. This makes it possible to delay the encounter of the two forks with each other so that replication fork movement can be interrogated (Fig 1D)[21,23,27]. Notably, there is no known upper limit to the size of the LacR barrier: any given *lacO* array can be increased in size by cloning in 4–48 *lacO* sequences (115–1490 bp) from other *lacO* plasmids (Table 2)[13,18]. Inhibition of DNA polymerases *prior* to release of the barrier results in replication fork 'uncoupling'[24] (Figs 1E, 2A, 2B lanes 3, 6), which causes fork reversal and nascent strand degradation[24], allowing these processes to be studied. The barrier can also be placed adjacent to site-specific DNA damage. This setup can be used to control whether replication forks encounter the lesion on the leading strand (Fig 1F) or lagging strand (Fig 1G)[17,19,22,28]. For DNA lesions that impact both DNA strands, such as a DNA interstrand cross-link[29], the barrier can also be used to control whether the lesion is encountered by a single replication fork (Fig 1H) or two replication forks (Fig 1I)[18,20]. Altogether, the reversible replication barrier allows various aspects of DNA replication and replication-coupled DNA repair to be studied.

In this manuscript, we describe protocols to control replication fork movement in *Xenopus* egg extracts using a LacR barrier. We also include assays to monitor DNA intermediates of DNA replication and use DNA structures that arise following replication fork uncoupling[24] (Fig 1E). Additionally, the reversible replication barrier can be combined with other approaches that are now standard for *Xenopus* egg extracts. Recent examples that have been applied in the context of the reversible replication barrier include

immunodepletion and rescue of target proteins[27,30–32], addition of small molecule inhibitors to interfere with protein function[24,26,27], chromatin immunoprecipitation[18,28,33,34], and chromatin capture[13,33]. The reversible replication barrier should also be compatible with recently-developed chromatin mass spectrometry approaches[26,35,36]. To date, we have only applied the LacR barrier to studies of DNA replication and replication-coupled DNA repair using *Xenopus* egg extracts. However, the approach should be readily applicable to any other *in vitro* system to study DNA replication using plasmid DNA templates[6,7,37]. Thus, the LacR barrier should be applicable to a broad range of additional topics, including chemotherapeutics[23]; sister chromatid cohesion[38]; repetitive DNA sequences[39]; G-quadruplexes[28]; chromatin biology[40]; telomeres[41]; checkpoints[42]; and structural biology[43,44].

Comparison to other methods

To our knowledge, there are no other established *in vitro* protocols that allow for replication forks to be stalled at a specific location and then seamlessly restarted. DNA interstrand cross-links (ICLs) can block DNA replication *in vitro*[29] but are not reversible and activate specific replication-coupled DNA repair pathways[20,32], so they are not ideal for studying replication-coupled DNA repair. The bacterial Tus-Ter system forms a long-lived replication block[45–48] but also has the drawback of activating DNA repair[49]. Multiple DNA-protein cross-links (DPCs) can also block DNA replication *in vitro*[22], but this also elicits DNA repair pathways[19,50]. Biotin-streptavidin linkages have not been reported to activate the same DNA repair pathways but are inefficient blocks to DNA replication[51]. Thus, the LacR barrier is a potent and reversible block to DNA replication that does not appear to elicit DNA repair pathways.

Limitations

The main limitation of the LacR barrier approach is that it has only been applied to *in vitro* DNA replication using the *Xenopus* egg extract system. However, the LacR barrier is functional when replication is studied using purified proteins[52] and also in mammalian cells[53,54]. Thus, we anticipate that the reversible replication barrier should be readily extendable to other systems.

We have gained valuable insights into vertebrate DNA replication using ~500–1000 base pair *lacO* arrays[13,18,23,24,26,27]. However, there may be different requirements for replication of longer stretches of DNA. The largest size *lacO* array described in this protocol is ~1500 base pairs (Table 2). Importantly, this is similar to the ~3000 base pairs replicated during chromosomal DNA replication in *Xenopus* egg extracts[10]. We also note that valuable insights into replication fork function have been gleaned from analyzing replication of ~3000 base pairs using purified yeast proteins[8]. Thus, we do not believe that the size ~500–1500 base pair *lacO* arrays described in this protocol is inherently limiting.

Overview of the procedure:

In this manuscript, we describe generalized protocols for studying DNA replication and use site-specific replication fork uncoupling as an example[24].

The procedure can be summarized as follows:

- Procedure 1 (Steps 1–147, Fig. 3 and 4), Preparation of the starting materials: in this section, we describe the preparation and purification of the biotinylated LacR repressor (Steps 1–63) as well as the preparation of plasmid DNA (Steps 64–120). We also explain how to create site-specific DNA damage in plasmid DNA by insertion of a chemically-modified oligonucleotide (Steps 121–147).
- Procedure 2 (Steps 1–25), Experimental approaches: in this section, we discuss methods to study in vitro DNA replication using LacR as a reversible replication barrier (Steps 1–15) as well as methods for the separation of replication intermediates (Steps 16–25).
- Procedure 3 (Steps 1–76), Analytical gels: in this section we describe the use of analytical agarose gels for the separation of replication fork structures using native agarose gels (Steps 24–27) or 2D native agarose gels (Steps 28–55), and the separation of nascent strands using denaturing agarose gels (Steps 56–76).
- Procedure 4 (Steps 1–19), Data acquisition and analysis: in this section, we provide detailed guidelines on how to perform phosphorimaging (Steps 1–7) and data analysis using Image J software (Steps 8–19).

The starting materials biotinylated LacR and template DNA) are largely the same between approaches. However, all other steps should generally be tailored to the specific questions under investigation and based on the exact DNA template used (in particular the *lacO* array size and presence of DNA damage), reaction compositions, analytical assays, and quantification approaches. Key considerations for all these steps are outlined in the ‘Experimental design’ section.

Experimental design

Starting materials: The only starting materials required for this protocol are the *Xenopus* egg extracts needed for plasmid DNA replication *in vitro*. High Speed Supernatant (HSS) is first used to load pairs of MCM2–7 complexes onto the DNA (‘licensing’)[55,56]. Subsequent addition of Nucleoplasmic Extract (NPE) converts MCM2–7 complexes to the active CMG helicase, which coordinates the assembly of the replication machinery (‘initiation’)[7]. Preparation of these extracts is described in other high-quality protocols[30,57]. The expertise needed to produce *Xenopus egg* extracts and the need fo*Xenopus* colony may make it challenging to produce egg extracts. However, the extracts used in this protocol (High-Speed Supernatant[58] and Nucleoplasmic Extract[7]) are stable for years when stored at −80°C and can be shipped on dry ice without loss of activity. Thus, it is also possible to obtain the relevant extracts through collaboration or by visiting a *Xenopus* lab with the appropriate expertise. Additionally, the protocols here should be applicable to other *in vitro* replication systems that use DNA templates derived from circular plasmid DNA[6,7,37].

Preparation of purified LacR—Purified LacR is needed to assemble a LacR barrier. To obtain this, we express biotinylated LacR in bacteria, then fractionate the lysates and use a monomeric avidin column to isolate the biotinylated LacR by affinity purification. We express biotinylated LacR in a 1 L culture then split this culture and freeze two cell

pellets prior to protein purification. One cell pellet (equivalent to 500 ml of culture) is used for purification and typically results in ~0.5–1.0 mg of biotinylated LacR, which is enough to last a single investigator for several years. The remaining cell pellet can be used for subsequent purifications. Because our approach is optimized for purification of a 500 ml culture and produces a substantial quantity of purified LacR we do not recommend scaling up the expression or purification.

DNA template preparation—In principle, essentially any circular DNA template can be replicated in *Xenopus* egg extracts[7]. We typically favor DNA templates of approximately 3–5 kilobase pairs (kbp) in size because plasmids in this size range separate well under standard analytical gel conditions (e.g., ~1% (w/v) agarose in TBE), and this size range limits most plasmids to a single initiation event[26,59]. Plasmid DNA is purified by phenol:chloroform extraction and ethanol precipitation to ensure that only highly pure DNA is used for experiments. This is particularly important to ensure consistency in the efficiency of DNA replication between different preparations of plasmid DNA. These considerations apply to all experiments involving *Xenopus* egg extracts, not just those involving a LacR replication barrier.

To control replication fork progression using a LacR replication barrier, purified LacR must be incubated with plasmid DNA harboring a *lacO* array. A variety of plasmids containing different size *lacO* arrays have been described[13,19,21,24] but we typically find that the plasmids described in this manuscript (Table 2) are suitable for most purposes. Plasmid DNA harboring a *lacO* array is susceptible to expansion and contraction (e.g. Fig 4B, lane 5). These rearrangements will alter the potency of the LacR block (stronger or weaker for expansions or contractions, respectively) and also increase heterogeneity of replication intermediates and products. It is therefore important to screen for subclones harboring rearrangements so they can be avoided. To this end, we typically purify DNA from multiple minipreps to limit population doublings. We also screen individual minipreps to confirm that the DNA has not undergone any rearrangements (Fig 4, lanes 5–12). Only small amounts of DNA are required (~150–300 ng per experiment) so these additional purification and screening steps only minimally impact the workflow.

Inclusion of DNA damage—The encounter of a single replication fork with DNA damage (Fig 1F–H) can also be studied by engineering DNA damage adjacent to a *lacO* array. This approach we describe here can be used to study any base modification (e.g. DNA-protein cross links[19], oxidized or methylated bases, cyclobutane pyrimidine dimers, abasic sites) by ligation of a modified DNA oligonucleotide into a *lacO* plasmid between two site-specific DNA nicking sites (e.g. in Fig 5 and[60]). However, lesions that involve both DNA strands (e.g. interstrand cross-links[29]) or are ordinarily repaired quickly prior to the onset of DNA replication (e.g. single-strand breaks[17]) require more complicated approaches. Regardless of the exact type of damage, we typically use plasmids containing a ~3 kbp ‘backbone’ (the region that does not contain the *lacO* array) and position the DNA damage ~0.3 kbp away from the inserted *lacO* array. Sites of replication initiation are stochastically determined and evenly distributed across plasmid DNA[59], so this positioning ensures that

most replication forks (~90%) encounter the DNA damage in a single orientation and on a specific strand (Fig 1F–G).

lacO array plasmid selection—A variety of *lacO* array plasmids ranging from ~0.5 kbp (e.g., *lacO*x16) to ~1.5 kbp (e.g., *lacO*x48) have been generated (Table 2). Selecting the appropriate array size involves balancing the need to block fork movement with the importance of synchronizing fork movement. Larger LacR barriers (e.g. 990 bp for *lacO*x32 and 1490 bp for *lacO*x48) will generally block replication forks for longer, which is useful for studying prolonged stalling or preventing the arrival of an opposing fork (Fig 1B)[18,19,21]. However, fork movement is highly variable[10], so larger arrays will also increase the asynchrony of termination events that are induced once the LacR barrier is disrupted. Thus, smaller *lacO* arrays (e.g. 365 bp for *lacO*x12 and 490 bp for *lacO*x16) are favored for studying termination (Fig 1C)[13]. A comparison between multiple array sizes can provide information on the regulation of replication fork progression (elongation or stalling, Fig 1D, F). For example, increasing the size of the array increases the duration of replication fork progression (elongation or stalling, Fig 1D, F) but does not increase the duration of termination. Thus, inactivation of proteins or pathways that affect fork progression should exert more of an effect with increasing array size while perturbations that affect termination should be unaltered.

***In vitro* replication reactions—**The reaction composition for *in vitro* replication reactions is relatively standardized (Procedure 2). However, the duration of the reaction varies considerably. Replication fork uncoupling, replication-coupled DNA repair, or replication fork stalling typically proceed for ~2–4 hours because these processes are relatively slow[19,24,29]. In contrast, termination of replication forks within a LacR array typically occurs within 5 minutes from when the LacR barrier is disrupted[13,26]. Thus, the timing of sample collection should be adjusted according to the process being studied. In addition to the time points collected, the role of different proteins can be assessed using small molecule inhibitors or by immunodepletion of target proteins, as described elsewhere. Finally, it can be beneficial to include a control plasmid that does not contain a *lacO* array (pCTRL in Fig 6A, e.g. pJD145 in Table 2) to serve as a loading control for subsequent analytical assays.

Analytical assays—In this manuscript, we describe a range of analytical assays that can be used to analyze replication intermediates (Procedure 3). Native gel analysis of replication intermediates can be performed with minimal sample processing and provides a wealth of information (Fig 6). Thus, we typically perform an analysis of unprocessed replication intermediates for all experiments. However, replication intermediates can contain multiple topoisomers of different DNA structures (e.g., θ and θ* in Fig 2 and Fig 6), which can make it difficult to unambiguously assign the identity of individual DNA species. In these situations, we purify and digest the DNA intermediates using restriction enzymes so that DNA species containing replication forks can be distinguished from fully unwound monomers (Fig 7). If the exact DNA structure of the replication fork containing DNA needs to be determined, then 2-dimensional gel electrophoresis (‘2D gels’) can be performed (Fig 8). In some cases, it is important to assay changes that do not alter the overall replication

fork structure. For example, the structures formed when a replication fork encounters a DNA lesion are essentially the same as those formed once the fork has bypassed the DNA lesion (compare Fig 1B and Fig 1F). In this situation, it is important to analyze the size of the nascent DNA strands by denaturing alkaline gel electrophoresis (Fig 9) so that the extension of nascent DNA strands beyond the lesion can be monitored. Denaturing gels can also be used to monitor the onset of degradation of nascent DNA strands with a high level of sensitivity because small amounts of degradation result in an appreciable change in the size of the nascent DNA strands (see LWS in Fig 9).

Data quantification—Densitometry provides a highly quantitative approach to measure changes in the abundance of different DNA species in different lanes of an analytical gel. In this manuscript, we include specific examples of how to quantify different processes that arise from nascent strand degradation, but in principle, any observable changes in the abundance of DNA species can be quantified. Typically, quantified data are normalized according to total lane signal, as this corrects for technical errors in loading and DNA purification. This normalization is particularly useful for monitoring the conversion of one or more precursors to one or more products. However, this approach may not be appropriate when one or more DNA species are degraded (e.g., during nascent strand degradation following replication fork uncoupling as in Fig 7). Thus, as an alternative, we normalize to a control plasmid that does not contain a *lacO* array and is rapidly fully replicated ('CTRL' in Figs 6 and 7).

MATERIALS

- **Biological material: *E. coli* strains**
  - T7 Express *E. coli* cells (NEB, cat. no. C2566H)
  - DH5α *E. coli* for subcloning (Thermo Scientific, cat. no. EC0111)

Reagents

General reagents:

- Bacto tryptone (Thermo Fisher Scientific, cat. no. 211705)
- Yeast extract (Thermo Fisher Scientific, cat. no. BP1422–500)
- Sodium chloride (NaCl) (Fisher BioReagents, cat. no. FLBP35810)
- Bacto agar (BD Bacto, cat. no. BD 214010)
- Ampicillin sodium salt (AMP) (Fisher BioReagents, cat. no. BP1760–25)
- Chloramphenicol (CAM) (Thermo Scientific Chemicals, cat. no. 227920250)
- Super optimal medium with catabolic repressor (SOC) medium (Invitrogen, cat. no. 15544034)
- Isopropyl β-D-1-thiogalactopyranoside (IPTG): Research Products IPTG (Research Products International, cat. no. 367-93-1) is used in Procedure 1, while Invitrogen IPTG (Invitrogen, cat. no. 15529019) is used in Procedure 2.

CRITICAL Research Products IPTG is used for most applications because it is more economical. Invitrogen IPTG is used to disrupt LacR barriers during replication reactions because we have empirically determined that it is more potent for this application and has a longer shelf life in liquid form.

- Biotin (Sigma Alrdich, cat. no. B4501)
- Sucrose (Invitrogen, cat. no. 15503022)
- Ethylenediaminetetraacetic acid (EDTA) (Thermo Scientific Chemicals, cat. no. 011931–36)
- Tris base (Fisher BioReagents, cat. no. FLBP15210)
- Complete protease inhibitor (Roche, cat. no. 11873580001)
- Dithiothreitol (DTT) (Bio-Rad, cat. no. 1610611)
- Polymin P (Themo Scientific Chemicals, cat. no. 178571000)
- SoftLink monomeric avidin resin (Promega, cat. no. V2012)
- Lysozyme (Sigma Aldrich, cat. no. L4919)
- Non-ionic detergent Brij58 (Thermo Scientific Chemicals, cat. no. 344295000)
- Ammonium sulfate (Sigma Aldrich, cat. no. A2939)
- Glycerol (Fisher BioReagents, cat. no. BP229–1)
- NuPAGE 4–12% Bis Tris SDS gels (Invitrogen, cat. no. NP0323BOX)
- NuPAGE MOPS SDS running buffer (Invitrogen, cat. no. NP001)
- Prestained protein ladder (Thermo Scientific, cat. no. 26617)
- Bovine serum albumin (BSA) (Sigma Aldrich, cat. no. A7906)
- Hydrochloric acid (HCl) (Fisher Chemical, cat. no. A144–500)
- QIAprep spin miniprep kit (Qiagen, cat. no. 27106)
- *Sac*I-HF (NEB, cat. no. R3156S)
- *Kpn*I-HF (NEB, cat. no. R3142S)
- rCutsmart buffer (10X) (NEB, cat. no. B6004S)
- Agarose: Hoefer agarose (Hoefer Inc, cat. no. GR140500X) and SeaKem GTG agarose (Lonza Inc, cat. no. 50074) CRITICAL: Hoefer agarose is used for most applications because it is more economical. SeaKem agarose is used for 2D native gels to ensure high quality.
- 1 kb DNA ladder (NEB, cat. no. N3232S)
- Ficoll (Sigma Aldrich, cat. no. F9378)
- Bromophenol blue (Fisher Chemical, cat. no. B392–5)
- Boric acid (Thermo Scientific Chemicals, cat. no. 012680-A7)

- SYBR gold nucleic acid gel stain (Invitrogen, cat. no. S11494)
- Ethanol (Fisher BioReagents, cat. no. BP2818100)
- Phenol/chloroform/isoamyl alcohol (25:24:1) (Thermo Scientific Chemicals, cat. no. 327115000)

  CAUTION Phenol/chloroform/isoamyl alcohol is toxic. Handle phenol/chloroform/isoamyl alcohol in a chemical fume hood.
- Chloroform/isoamyl alcohol (24:1) (Thermo Scientific Chemicals, cat. no. 327155000)

  CAUTION Chloroform/isoamyl alcohol is toxic. Handle chloroform/isoamyl alcohol in a chemical fume hood.
- 5’ phosphorylated, HPLC/PAGE-purified oligonucleotide containing the DNA adduct of interest
- Nb.*Bsm*I (NEB, cat. no. R0706S)
- T4 DNA ligase (NEB, cat. no. M0202S)
- Monarch® PCR & DNA Cleanup Kit (5 μg) (NEB, cat. no. T1030L)
- Monarch® Plasmid Miniprep Columns (NEB, cat. no. T1017L)
- NEBuffer r3.1 (10X) (NEB, cat. no. B7203S)
- *Age*I-HF (NEB, cat. no. R3552S)
- T5 Exonuclease (NEB, cat. no. M0663S)
- Ethidium bromide (Invitrogen, cat. no. 15585011)

  CAUTION Ethidium bromide is a mutagen. Wear proper personal protective equipment (PPE) and follow all chemical safety procedures when handling ethidium bromide.
- Sodium dodecyl sulfate (SDS) (Sigma Aldrich, cat. no. L3771)
- Sodium acetate (Sigma Aldrich, cat. no. W302406)
- Potassium chloride (KCl) (Fisher BioReagents, cat. no. BP366–500)
- Magnesium chloride hexahydrate ($MgCl_2$-$6H_2O$) (Thermo Scientific Chemicals, cat. no. 447155000)
- HEPES (Fisher BioReagents, cat. no. BP310–1)
- Potassium hydroxide (KOH) (Spectrum Chemical Manufacturing Corporation, cat. no. PO180500GM)
- Adenosine 5’-triphosphate disodium salt hydrate (ATP) (Sigma Aldrich, cat. no. A7699)
- Sodium hydroxide (NaOH) (Sigma Aldrich, cat. no. 221465)
- Phosphocreatine (PC) (Sigma Aldrich, cat. no. P7936)

- Potassium phosphate dibasic (K_2HPO4) (Thermo Fisher Scientific, cat. no. P288500)
- Potassium phosphate monobasic (KH_2PO4) (Thermo Scientific Chemicals, cat. no. 205925000)
- Creatine Phosphokinase (CPK) (Sigma Aldrich, cat. no. C3755) for ATP regeneration
- *Xenopus* egg extract: High-speed supernatant (HSS) (prepared as described[30])
- *Xenopus* egg extract: NucleoPlasmic extract (NPE) (prepared as described[30])
- Nocodazole (Sigma Aldrich, cat. no. M1404)
- [α-^{32}P]-dATP (3000 Ci/mol) (Revvity, cat. No. NEG012H250UC)
- Ribonuclease A (RNAse) (Sigma Aldrich, cat. no. R4642)
- Proteinase K (Roche, cat. no. 03115879001)
- DNA polymerase inhibitor Aphidicolin (Sigma Aldrich, cat. no. A0781–5MG)
- Dimethyl sulfoxide (DMSO) (Sigma Aldrich, cat. no. D8418)
- Glycogen (Roche, cat. no. 10901393001)
- *Xmn*I (NEB, cat. no. R0194S)
- *Alw*NI (NEB, cat. no. R0514S)
- Trichloroacetic acid (TCA) (Thermo Fisher Scientific, cat. no. FLA322500)
- *Xho*I (NEB, cat. no. R0146S)
- *Dra*III-HF (NEB, cat. no. R3510S)
- Loading dye: Bromocresol green (Fisher Chemical, cat. no. B383–5)
- Loading dye: Xylene cyanol (Thermo Scientific Chemicals, cat. no. 422690050)

  Plasmids: pBirAcm, pET11a[lacI::avi] (pJD72 from[13]), pJD87, pJD91, pJD100, pJD145, pJD152, pJD156, pJD161, pJD194, pJD195

  CRITICAL pBirAcm is available from Avidity. All other plasmids are available upon request from James Dewar. Details of the plasmids used in this protocol are listed in Table 2.

Oligonucleotides: SDO1 (CATTCACCGGTATCCTTACGAGCG)

Critical Step Base modifications are made within the *Age*I site (ACCGGT) of SDO1 so plasmids containing the modified oligonucleotide will be resistant to *Age*I digestion (in Procedure 1: Step 132).

SDO2 (CATTCACTGGTATCCTTACGAGCG)

SDO2 is identical to SDO1 except for a single base pair mismatch within the *Age*I site (AC*T*GGT). This oligonucleotide can be used as a positive control for introduction of a modified oligonucleotide in Procedure 1: steps 127–147

Equipment

- Petri dishes (Fisherbrand, cat. no. FB0875713)
- 15 ml falcon culture tubes (Falcon, cat. no. 352059)
- 5 ml tubes (Eppendorf, cat. no. 0030122321)
- 15 ml conical tubes (Falcon, cat. no. 352097)
- 50 ml conical tubes (Corning, cat. no. 430828)
- 0.5 ml Eppendorf safe-lock microcentrifuge tubes (Eppendorf, cat. no. 022363611).

  CRITICAL The phenol/chloroform extraction of DNA described in this protocol is optimized for use with these tubes. Use of alternative tubes may result in reduced DNA yield or purity.
- 0.6 ml Axygen microcentrifuge tubes (Axygen, cat. no. MCT060CS)
- 1.7 ml microcentrifuge tubes (Corning, cat. no. 3620)
- 2 ml microcentrifuge tubes (VWR, cat. no. 87003–298)
- 20 ml glass beaker (Corning, cat. no. 1000–20)
- 500 ml 0.2 μm filter unit (Thermo Scientific, cat. no. 4500020)
- 1 L 0.2 μm filter unit (Thermo Scientific, cat. no. 1270020)
- 10 ml syringe (BD, cat. no. 309695)
- 50 ml syringe (BD, cat. no. 309653)
- 0.2 μm syringe filter (Sartorius, cat. no. 16541K)
- 1 L glass bottle (Fisherbrand, cat. no. FS143951000)
- 1 L glass flask (PYREX, cat. no. 49801L)
- 250 ml glass flask (PYREX, cat. no. 4980250)
- 500 ml plastic beaker (Fisherbrand, cat. no. 3264950500)
- 1 L plastic beaker (Fisherbrand, cat. no. 3264951000)
- 3 L plastic beaker (Fisherbrand, cat. no. 3264953000)
- Water bath (Fisher Scientific)
- New Brunswick Scientific Innova incubator shaker (Eppendorf)
- Incubator for bacteria culture (VWR)
- UV-Visible spectrophotometer (Thermo Scientific)

- Cuvettes (VWR, cat. no. 97000–586)
- Superspeed floor centrifuge (Thermo Scientific)
- Fiberlite 4 × 1000 ml fixed angle rotor (Thermo Scientific, cat. no. 096–041075)
- Fiberlite 1000 ml bottles (Thermo Scientific, cat. no. 010–1491)
- Eppendorf 5430R microcentrifuge (Eppendorf)
- 15/50 ml rotor for Eppendorf 5430R microcentrifuge (Eppendorf, cat. no. 022654306)
- 1.7 ml × 30 rotor for Eppendorf 5430R microcentrifuge (Eppendorf, cat. no. 022654047)
- Incubator for digestion (VWR)
- Low speed orbital shaker (Corning)
- Probe sonicator (Fisher Scientific)
- Stir bar (Fisherbrand)
- Stir plate (VWR)
- Chromatography columns (Bio-Rad, cat. no. 7311550)
- SDS-PAGE gel tank (Invitrogen)
- Slide-A-Lyzer Dialysis Cassette 20 kilodalton (kDa) molecular weight cut off (MWCO) (Thermo Scientific, cat. no. A52976)
- Nanodrop Spectrophotometer (Thermo Fisher Scientific)
- Amicon Ultra-0.5 Centrifugal Filter Unit, 10 kilodalton (kDa) molecular weight cut off (MWCO) (Millipore, cat. no. UFC5010)
- Electrophoresis power supply (Fisher Scientific)
- Gel electrophoresis systems (CBS Scientific, cat. no. GCMHU-602 & GCHSU-020)
- UV transilluminator (Bio-Rad)
- 0.2 μm nitrocellulose membrane (Bio-Rad, cat. no. 1620112)
- Hybond N+ membrane (Cytiva, cat. no. RPN303B)
- Whatman paper (Cytiva, cat. no. 3030917)
- Gel drier (Hoefer Scientific)
- Vacuum pump (Fisher Scientific)
- Vacuum line/aspirator
- Fluorescent flexible ruler (UVP, cat. no. 85000301)
- GE Amersham Typhoon Phosphor imager (GE)

- Phosphorimager screen (GE)
- Exposure cassette (GE)
- Light box for phosphorimager screens
- Microwave (Frigidaire)
- pH meter (Fisher Scientific)
- pH strips (Fisherbrand, cat. no. 13-640-516)
- Vortex (Fisher Scientific)
- Tube rotator (Thermo Scientific)
- Analog block heater (VWR)
- Amicon Ultra-0.5 Centrifugal Filter Unit, 100 kilodalton (kDa) molecular weight cut off (MWCO) (Millipore, cat. no. UFC510008)
- Ultra-fine tips (Eppendorf, cat. no. 22351656)
- BrandTech Pipettors.

  CRITICAL This protocol involves pipetting small volumes of viscous liquids. Many pipettors are not able to do this with sufficient accuracy and precision for highly reproducible results. We have found BrandTech Pipettors to be the most accurate and reproducible for pipetting small volumes, particularly of viscous liquids. Other types of pipettors can be used but our experience is that these will introduce unnecessary variability and inaccuracy.
- BrandTech P20 tips (BrandTech BRAND, cat. no. 732204).

  CRITICAL We recommend using BrandTech pipette tips as we have found them to be the most accurate.
- X-acto knife
- Milli-Q ultrapure water system
- Software:
- ImageJ (https://imagej.net/ij/download.html)
- Excel

Reagent setup

Lysogeny Broth (LB) medium—Add 100 ml of Milli-Q H_2O to a 1 L glass bottle. Add 5 g Bacto tryptone, 2.5 g yeast extract, and 5 g NaCl. Add Milli-Q H_2O up to 0.5 L. During the addition of Milli-Q H_2O, rinse the inside wall of the bottle to ensure that all material is collected at the bottom of the bottle. Autoclave at 121°C for 45 min to sterilize. Store at room temperature (approximately 22°C) for up to 4 months after autoclaving.

LB agar plates containing AMP, CAM, and/or IPTG—Add 100 ml of Milli-Q H_2O to a 1 L glass flask. Add 7.5 g Bacto agar, 5 g Bacto tryptone, 2.5 g yeast extract, and 5 g

NaCl. Add Milli-Q H_2O up to 0.5 L. During the addition of Milli-Q H_2O, rinse the inside wall of the flask to ensure that all material is collected at the bottom of the flask. Add a stir bar. Autoclave at 121°C for 45 min to sterilize. Mix gently on a stir plate for ~30 min until the medium reaches 50–60°C. Add AMP to 100 μg/ml, CAM to 17 μg/ml (in ethanol), and/or IPTG to 1.1915 mg/ml. Mix for 1 min. Pour ~20 ml per petri dish. Incubate on a bench until solidified or overnight. Store at 4°C for up to one month.

LB/AMP/CAM medium—Add 20 ml of LB medium to a sterile 250 ml glass flask. Add AMP (50 mg/ml in H_2O) to 100 μg/ml and CAM (34 mg/ml in ethanol) to 17 μg/ml. Make fresh for each use.

Tris-HCl (1 M, pH 8.0 or 7.5)—Add 121.1 g tris base to a 1 L plastic beaker. Add Milli-Q H_2O up to 800 ml. Mix on a stir plate until dissolved. Using a pH meter, adjust the pH to the desired value (pH 8.0 or pH 7.5) by adding concentrated HCl. Add Milli-Q H_2O up to 1 L. Sterilize by filtering using a 1 L 0.2 μm filter unit. Store at room temperature for up to one year.

EDTA (0.5 M)—Add 73.06 g of EDTA to a 3 L plastic beaker. Add Milli-Q H_2O up to 400 ml. Mix on a stir plate while adding 29 g NaOH pellets. While mixing, slowly add another 1–2 g of NaOH pellets until all EDTA dissolves. Sterilize by filtering using a 500 ml 0.2 μm filter unit. Store at room temperature for up to one year.

CAUTION This is an exothermic reaction. Do not make it in a glass beaker as it may shatter from the heat.

Complete (25X)—Add 1 tablet of complete to a 5 ml conical tube. Add Milli-Q H_2O up to 2 ml. Rotate end over end and periodically vortex until dissolved. Make fresh for each use.

Freezing buffer—Add 5 g of sucrose, 500 μl of EDTA (0.5 M), 2.5 ml of Tris-HCl (1M, pH 7.5), and 2 ml of complete (25X) to a 50 ml conical tube. Add Milli-Q H_2O up to 50 ml. Rotate end-over-end at room temperature until dissolved. Sterilize by filtering into a new 50 ml conical tube using a 50 ml syringe attached to a 0.2 μm filter. Make fresh for each use and keep on ice.

Brij58 (10% w/v)—Brij58 is supplied as small pellets so the total volume prepared will depend on the size of the individual pellet used. First, weigh out 0.075–0.125 g of Brij58 in a 1.5 ml tube and record the exact amount added. Next, add water to 0.75 ml then mix by end over end rotation until dissolved. Finally, add additional water to bring the final concentration to 10% (w/v) depending on the amount of Brij58 originally weighed out. Store at 4°C for up to 2 days.

Buffer A (4X)—Prepare the day prior to purification of LacR as follows: add 200 ml of Tris-HCl (1M, pH 7.5), 8 ml of EDTA (0.5 M), and 23.38 g of NaCl to a 1 L plastic beaker. Add Milli-Q H_2O up to 1 L. Mix on a stir plate until dissolved. Sterilize by filtering using a 1 L 0.2 μm filter unit. Store at 4°C overnight. On the day of the LacR purification, add 617

mg of fresh DTT and mix by inversion to dissolve the DTT. Make fresh for each use and keep on ice.

Buffer 1—Add 2.5 ml of Buffer A (4X), 1 g of sucrose, 80 µl of EDTA (0.5 M), and 400 µl of complete (25X) to a 15 ml conical tube. Add Milli-Q H_2O up to 10 ml. Rotate end-over-end at room temperature until dissolved. Make fresh for each use and keep on ice.

Extraction buffer—Add 2.5 ml of Buffer A (4X), 80 µl of EDTA (0.5 M), 400 µl of complete (25X), 1.8 ml of NaCl (5 M), and 300 µl of 1M IPTG to a 15 ml conical tube. Add Milli-Q H_2O up to 10 ml. Rotate end-over-end at room temperature until dissolved. Make fresh for each use and keep on ice.

Wash buffer—Add 5 ml of Buffer A (4X), 800 µl of complete (25X), and 2.922 g of NaCl to a 50 ml conical tube. Add Milli-Q H_2O up to 20 ml. Rotate end-over-end at room temperature until dissolved. Make fresh for each use and keep at 4°C.

Buffer 2—Add 2.5 ml of Buffer A (4X) to a 15 ml conical tube. Add Milli-Q H_2O up to 10 ml. Rotate end-over-end at room temperature until dissolved. Make fresh for each use and keep at 4°C.

Elution buffer—Add 5 ml of Buffer A (4X) to a 50 ml conical tube. Add Milli-Q H_2O up to 20 ml. Add 24.44 mg of biotin. Rotate end-over-end at room temperature until dissolved. Make fresh for each use and keep at 4°C.

Dialysis buffer—Add 750 ml of Buffer A (4X), 8.77 g of NaCl, and 1.15 L of glycerol to a 3 L plastic beaker. Add Milli-Q H_2O up to 3 L. Mix on a stir plate until dissolved. Sterilize by filtering using a new 1 L 0.2 µm filter unit for every liter of buffer. Make fresh for each use and keep at 4°C.

DNA loading dye (6X, −/+ SDS)—Add 1.5 g of Ficoll, 1.33 ml of EDTA (0.5 M), 200 µl of Tris-HCl (1M, pH 8.0), and 9 mg of bromophenol blue to a 15 ml conical tube. Add 50 µl of SDS (20% w/v) as appropriate. Add Milli-Q H_2O up to 10 ml. Rotate end-over-end at room temperature until dissolved. Sterilize by filtering into a new 15 ml conical tube using a 10 ml syringe attached to a 0.2 µm filter. Store at room temperature for up to one year.

TBE buffer (10X)—Add 108 g of tris base, 55 g of boric acid, and 50.91 ml of EDTA (0.5 M) to a 1 L plastic beaker. Add Milli-Q H_2O up to 1 L. Mix on a stir plate until dissolved. Filter sterilize using a 1 L 0.2 µm filter unit. Store at room temperature for up to one month.

CRITICAL TBE (10X) should not be used if it shows signs of precipitation because this will negatively impact electrophoresis. Filter TBE (10X) immediately after it is made to minimize subsequent precipitation and keep for no longer than one month for best results.

Agarose gel (1% (w/v) in TBE (1X))—Combine 1.2 g of agarose, 12 ml of TBE buffer (10X), and 108 ml of water in a conical flask. Weigh the mixture and then microwave until all agarose is melted. Weigh the mixture once more, then add an appropriate volume of

water to account for the water loss due to evaporation Stir constantly with a stir bar and allow to cool to ~55°C before pouring the mixture into the casting tray and placing a comb. 20. After the gel has cooled and solidified, submerge the gel in an electrophoresis tank containing TBE buffer (1X).

(Optional) for UV visualization of DNA, or to for separation of DNA molecules by shape, add ethidium bromide (10 mg/ml) to a final concentration of 0.3 mg/ml.

(Optional) scale the amount of agarose and the total gel volume up or down as needed to match the gel composition needed.

CRITICAL TBE (10X) should be stored no longer than a month and re-made if signs of precipitation are visible. It is recommended to filter TBE (10X) when it is made to minimize subsequent precipitation.

CRITICAL Addition of water after microwaving is necessary to ensure that gel percentage is accurate.

PAUSE POINT Agarose (1% w/v) gels submerged in TBE buffer (1X) can be stored at room temperature for up to 2 days.

Extraction Stop—Add 50 µl of Tris-HCl (1 M, pH 7.5), 50 µl of EDTA (0.5 M), 25 µl of SDS (20% w/v), and 875 ml of Milli-Q H_2O to a 1.7 ml microcentrifuge tube. Mix by pipetting up and down. Make fresh for each use.

Nocodazole (5 mg/ml)—Dissolve 10 mg of nocodazole in 2 ml of DMSO to make a 5 mg/ml solution. Transfer 50 µl aliquots to 0.5 ml tubes, then flash freeze in liquid nitrogen and store at −80°C for up to several years.

Nocodazole (0.5 mg/ml)—Thaw a 50 µl aliquot of nocodazole (5 mg/ml) at room temperature. Add 450 µl of DMSO then pix by pipetting and vortexing to make a 0.5 mg/ml solution. Transfer 25 µl aliquots to 0.5 ml tubes, then flash freeze in liquid nitrogen and store at −20°C for up to a year.

Egg lysis buffer (ELB) Salts (10X)—Add 5.08 g of $MgCl_2$-$6H_2O$, 37.28 g of KCl, and 100 ml of HEPES-KOH (1 M, pH 7.7) to a 1 L plastic beaker. Add Milli-Q H_2O up to 1 L. Mix on a stir plate until dissolved. Sterilize by filtering using a 1 L 0.2 µm filter unit. Store at 4°C for up to six months and protect from light.

Egg lysis buffer (ELB) (5X)—Add 25 ml of ELB salts (10X) and 21.4 g of sucrose to a 50 ml conical tube. Add Milli-Q H_2O up to 50 ml. Rotate end-over-end at room temperature until dissolved. Sterilize by filtering into a new 50 ml conical tube using a 50 ml syringe attached to a 0.2 µm filter. Store 0.5 ml aliquots at 4°C for up to one month.

Potassium phosphate buffer (10 mM, pH 7.0)—Add 61.5 µl of K_2HPO_4 and 38.5 µl of KH_2PO_4 to a 15 ml conical tube. Add Milli-Q H_2O up to 10 ml. Rotate end-over-end at

room temperature until dissolved. Sterilize by filtering into a new 10 ml conical tube using a 10 ml syringe attached to a 0.2 μm filter. Store at room temperature for up to 1 year.

PC (1 M)—Add 2.56 g of PC to a 15 ml conical tube. Add potassium phosphate buffer (10 mM, pH 7.0) up to 10 ml. Rotate end-over-end at room temperature until dissolved. Sterilize by filtering into a new 15 ml conical tube using a 10 ml syringe attached to a 0.2 μm filter. Store 50 μl aliquots at −20°C for up to six months.

ATP (0.2 M)—Add 0.551 g of ATP and 5 ml of Milli-Q H_2O to a 5 ml conical tube. Using pH strips, adjust the pH to 7.0 with approximately 150 μl of NaOH (10 M). Rotate-end-over end at room temperature briefly. Sterilize by filtering into a new 5 ml conical tube using a 10 ml syringe attached to a 0.2 μm filter. Store 50 μl aliquots at −20°C for up to six months.

CPK (5 mg/ml)—Add 5 mg of CPK, 10 μl of HEPES (1 M, pH 7.5), 10 μl of NaCl (5 M), and 1 ml of glycerol (50% v/v) to a 1.7 ml microcentrifuge tube. Rotate end-over-end at room temperature briefly. Sterilize by filtering into a new 1.7 ml microcentrifuge tube using a 5 ml syringe attached to a 0.2 μm filter. Store 50 μl aliquots at −20°C for up to six months.

ATP regenerating system (ARS)—Add 5 μl of ATP (0.2 M), 10 μl of PC (1 M), and 0.5 μl of CPK (5 mg/ml) to a 0.6 ml microcentrifuge tube. Mix gently by pipetting up and down. Prepare fresh and store on ice for up to 2 h before use.

IPTG (1 M in ELB (1X)) (used in experimental approaches)—To 1 g of IPTG stock, add 839 μl of ELB (5X), and 4.196 ml of Milli-Q H_2O. Rotate end-over-end at room temperature until dissolved. Divide the solution into 50 μl aliquots and flash freeze in liquid nitrogen. Store the aliquots at −20°C for up to 6 months.

Aphidicolin (98.5 mM)—Add 150 μl of DMSO directly to 5 mg of aphidicolin in the glass vial provided (i.e. the manufacturer's container). Rotate end-over-end at room temperature until dissolved. Prepare 3.5 μl aliquots and flash freeze in liquid nitrogen. Store the aliquots at −80°C for up to 1 year.

Aphidicolin Release Mix—Allow Aphidicolin (98.5 mM in DMSO) to thaw in room temperature water and then dilute to 30 mM in DMSO. We typically combine 3 μl Aphidicolin (98.5 mM) with 7 μl DMSO for ease of mixing. Next, dilute Aphidicolin to 12 mM in 40% (v/v) DMSO. We typically combine 10 μl Aphidicolin (30 mM in DMSO) with 15 μl water. Finally, prepare Aphidicolin Release mix (3.7 mM Aphidicolin, 700 mM IPTG, ELB (1X) in 12% (v/v) DMSO) by diluting Aphidicolin (32 mM in 40% (v/v) DMSO) with IPTG (1M in ELB (1X)). We typically combine 2 μl Aphidicolin (12 mM in 40% (v/v) DMSO) with 4.5 μl IPTG (1M in ELB (1X)).

CRITICAL Prepare any Aphidicolin dilutions and Aphidicolin release mix fresh each time and subaliquot immediately. Thoroughly mix each aphidicolin-containing solution by rapidly pipetting up and down 10–20 times.

IPTG (250 mM in ELB (1X))—Allow IPTG (1M in ELB (1X)) to thaw in room temperature water for at least 30 min then dilute to 250 mM in ELB (1X). We typically add 2 µl of IPTG (1M in ELB (1X)) to 4 µl of ELB (1X).

CRITICAL Incompletely thawed IPTG (1M in ELB (1X)) may not effectively disrupt the LacR barrier.

Alkaline loading buffer (6X, −/+ NaOH)—Add 618.56 µl of EDTA (0.5 M), 9.28 g of Ficoll, 77.3 mg of bromocresol green, and 128.9 mg of xylene cyanol to a 50 ml conical tube. Add Milli-Q H_2O up to 50 ml. Rotate end-over-end at room temperature until dissolved. Sterilize by filtering into a new 50 ml conical tube using a 10 ml syringe attached to a 0.2 µm filter. Store at room temperature for up to one year. Before use, add 15.46 µl of freshly made 10M NaOH to 500 µl of alkaline loading buffer and mix well by pipetting.

Alkaline buffer (10X)—Add 25 ml of NaOH (10 M) and 10 ml of EDTA (0.5 M) to a 500 ml plastic beaker. Add Milli-Q H_2O up to 500 ml. Mix on a stir plate until dissolved. Sterilize by filtering using a 500 ml 0.2 µm filter unit. Store at room temperature for up to 1 day. Make fresh before each use.

PROCEDURE 1: Preparation of materials • Timing 10.5 d

Purification of lac repressor (LacR) • Timing 4.5 d

Chemical transformation of plasmid containing LacR • Timing 2 h

1. Before transformation, complete the following steps:
   - Prepare LB/AMP/CAM agar plates.
   - Warm a water bath to 42°C.
2. Thaw 50 µl of chemically competent NEB T7 express *E. coli* cells on ice, and mix gently by pipetting up and down.
3. Add approximately 0.5 µl of pBirAcm and pET11a[lacl::avi] (Table 2), each at~20 ng/µl, to the bottom of a 15 ml falcon culture tube and place on ice. pET11a[lacl::avi] expresses LacR fused to an AviTag. pBirAcm expresses the BirA biotin ligase that biotinylates the AviTag.
4. Add 50 µl of NEB T7 Express cells to the falcon tube containing the plasmid mixture (from step 3). Mix by carefully swirling the tip of the pipet through the mix.
5. Incubate the plasmid-cell mixture on ice for 30 min.
6. Heat-shock the plasmid-cell mixture by incubating in a 42°C water bath for 10 sec.
7. Immediately place the plasmid-cell mixture back on ice and incubate for 5 min.
8. Add 950 µl of room temperature SOC medium into the tube containing the transformed cells.

9. Let the cells recover by incubating in a 37°C shaking incubator at 250 rpm for 60 min.

10. During the recovery period (step 9), warm LB/AMP/CAM Agar plates in a 37°C incubator.

11. After the recovery period (step 9), mix the cells gently by pipetting and perform a 10-fold dilution using SOC medium.

12. Spread 100 µl of undiluted cells on one half of the plate and 100 µl of 10-fold diluted cells on the other half of the plate.

13. Incubate the plate overnight (16 h) at 37°C.

14. After the overnight incubation (step 13), remove the plates from 37°C and store at 4°C.

    PAUSE POINT Store LB agar plates at 4°C for up to 2 days.

Biotinylated LacR Expression • Timing 20 h

15. From the plates from step 14, inoculate a single colony into 20 ml of LB/AMP/CAM medium. Grow it overnight (16 h) at 37°C with aeration.

16. Add 20 ml of the overnight culture to 1L of LB/AMP/CAM medium. Grow the bacteria at 37°C with aeration to OD_{600} 0.5–0.6.

17. Add IPTG (1 mM) and biotin (50 µM) to induce the expression and biotinylation of LacR. Incubate for 2 h at 37°C with aeration. Supplement with additional biotin (50 µM) at 60 min to ensure that LacR is fully biotinylated.

18. Transfer the culture to a 1L centrifuge tube and centrifuge it in a superspeed floor centrifuge at 4,000 × *g*, 4°C to pellet cells.

19. Remove and discard the LB media. Then, resuspend the pellet(s) in 20 ml of freezing buffer.

20. Flash-freeze two 10 ml aliquots of cell resuspension from step 19 in liquid nitrogen and store at −80°C until ready to use.

    PAUSE POINT Frozen cell resuspensions can be used immediately or stored at −80°C for several years prior to use.

Biotinylated LacR Purification • Timing 1 d

21. The day before purification, prepare the following:

    - Buffer A (4X, without DTT). Store at 4°C for up to 2 days.
    - Brij58 (10% w/v in water). Store at 4°C for up to 2 days.

22. The day of the purification, complete the following steps:

    - Cool a microcentrifuge to 4°C.

- Prepare a gravity flow column containing 1 ml Softlink monomeric avidin resin according to the manufacturer's instructions. Store at 4°C according to the manufacturer's instructions until ready to use.
- Freshly add DTT to Buffer A (4X).
- Prepare Buffer 1 and chill on ice.

23. Thaw the LacR cell resuspension (from step 20) on ice. Perform all subsequent steps on ice unless otherwise stated.

24. Centrifuge the cell resuspension at 4,000 × *g*, 4°C for 10 min in a microcentrifuge to pellet the cells. Alternatively, a high-speed floor centrifuge can be used for all centrifuge steps during the LacR purification.

25. During the centrifugation, prepare lysozyme (10 mg/ml in 20 mM Tris-HCl, pH 7.5).

26. Remove and discard the supernatant and resuspend the cell pellet from step 24 in 10 ml of Buffer 1 by pipetting.

27. Add 204 µl of lysozyme (10 mg/ml) and 102 µl of Brij 58 (10% w/v)

28. Incubate at room temperature for 30 min with end-over-end rotation to lyse the bacteria.

29. Split the suspension between five 2 ml microcentrifuge tubes and centrifuge at 14,000 × *g*, 4°C for 40 min to pellet the suspension.

30. During centrifugation, prepare the Extraction Buffer and chill on ice.

31. At the end of the centrifugation step, place the tubes on ice. Remove and collect the supernatants (soluble fraction) in a 15 ml conical tube then flash freeze in liquid nitrogen and store at −80°C.

CRITICAL STEP Most of the protein is associated with DNA in the insoluble fraction. The soluble fraction (supernatant) is therefore removed at this point to enhance the purity of the final product. However, this soluble fraction can be used later to yield a lower quantity of biotinylated LacR by performing steps 32–63.

32. Resuspend each pellet (insoluble fraction) from step 31 in 2 ml of Extraction Buffer and combine all the fractions in a new 15 ml conical tube (Fig 3A). Insoluble pellets will be viscous and hard to resuspend. To help resuspend the pellet, alternate between pipetting up and down and rotating end over end at 4°C.

33. Sonicate the cell suspension on ice for, as an example, 20 sec 3 times with 20 sec rest in between each sonication (Fig 3B lane 1).

CRITICAL STEP The sonication settings provided are those typically used in our laboratory. However, sonication conditions must be empirically determined for each sonicator.

34. Split the sonicated cell suspension between five 2 ml microcentrifuge tubes and centrifuge at 14,000 × *g,* 4°C for 40 min to pellet the cell suspension.

35. Pool the supernatants and transfer the solution to a 20 ml glass beaker (Fig 3B lane 2). Perform all subsequent LacR purification steps in a cold room at 4°C.

36. Slowly add 0.03–0.06 volumes of Polymin P (1% v/v) while stirring constantly with a stir bar to precipitate DNA.

37. After addition of Polymin P solution stir for an additional 30 min.

38. To facilitate the harvesting of the supernatant without disturbing the pellet, split the suspension between five 2 ml microcentrifuge tubes and centrifuge at 17,590 × *g,* 4°C for 20 min.

CRITICAL STEP Do no centrifuge faster than 17,590 RCF, as this can negatively impact the prep for reasons that are unclear.

39. Carefully remove the supernatant from each tube without disturbing the pellet (precipitated DNA). Combine all supernatants in a 20 ml glass beaker (typically ~6.5 ml).

40. Precipitate the LacR-containing protein fraction by slowly adding ammonium sulfate to the supernatant (step 39) to 37% saturation (corresponding to 0.22 g per ml of supernatant, Fig 3A). Stir constantly during ammonium sulfate addition. Meanwhile, prepare Wash Buffer and Buffer 2 and store at 4°C.

41. Once all of the ammonium sulfate has been added, stir for an additional 45 min or longer.

PAUSE POINT Ammonium sulfate can stir overnight.

42. Split the suspension between five 2 ml microcentrifuge tubes and centrifuge at 17,590 × *g* 4°C for 20 min to pellet the suspension.

43. Remove and discard the supernatants. To remove all supernatant, and the contaminants contained in it, remove all excess supernatant with a pipette, and then invert the tubes on a paper towel.

44. Resuspend each pellet (protein fraction) in 0.4 ml of Wash Buffer and combine into a single 2 ml suspension (Fig 3B lane 3).

45. Wash the Softlink monomeric avidin column with 10 ml of Buffer 2.

46. Apply 1 ml of the sample from step 44 to the column then cap the column to incubate for 30 min (Fig 3A). This allows the biotinylated LacR to bind to the monomeric avidin. While the column is incubating, prepare the Elution Buffer and store at 4°C.

47. Apply the remaining 1 ml of lysate to the column then cap the column to incubate for 30 min (Fig 3A). This allows the remaining biotinylated LacR to bind to the monomeric avidin.

48. After column binding is complete (step 47), collect the flowthrough and store at 4°C until SDS PAGE analysis (step 54).

49. Wash the column 3 times with 5 ml of Wash Buffer and, each time, collect and store the flowthrough (Fig 3A, 2B lanes 5–7) at 4°C until SDS PAGE analysis (step 54).

50. Apply 1 ml of Elution Buffer to the column. Incubate for 30 min to allow the biotin contained in the Elution buffer to displace the biotinylated LacR from the monomeric avidin.

51. Elute the first elution in a clean 1.5 ml tube.

52. Repeat steps 50–51 two more times to perform 2 additional elutions.

53. (Optional) Apply 1 ml of elution buffer to the column and incubate overnight to elute any remaining protein from the column. Collect overnight elution the next day (Fig 3B lane 11). This step can be performed to check that LacR was efficiently eluted in elutes 1–3 (steps 50–52), which should elute most of the protein.

CIRITICAL STEP We do not recommend using the overnight elution, as we typically find it is less active.

Biotinylated LacR Dialysis • Timing 20 h

54. Analyze the flowthrough (step 48), washes (step 49), and elutes (steps 50–53) using SDS-polyacrylamide gel electrophoresis (PAGE) to check that purification was successful (Fig 3B).

55. Pool elutes 1–3 (steps 50–52) together and pipette the volume into a Slide-A-Lyzer 20 kilodalton (kDa) molecular weight cut off (MWCO) dialysis cassette according to the manufacturer's instructions. Float the dialysis cassette in 1L of fresh dialysis buffer. Incubate the dialysis cassette at 4°C and stir for 1h on a stir plate to perform the 1st round of dialysis. Meanwhile, regenerate the Softlink column according to manufacturer's instructions.

56. Replace the buffer with 1 L of fresh dialysis buffer and perform a 2nd round of dialysis overnight (16 h) at 4°C, using the same setup as the 1st round of dialysis (step 55).

57. The following morning, replace the buffer with 1 L of fresh dialysis buffer and perform the 3rd round of dialysis for 1 h at 4°C, using the same setup as the 1st round of dialysis (step 55).

58. After the 3rd round of dialysis (step 57), flash freeze and store the dialyzed LacR protein and an aliquot of the 3rd round Dialysis Buffer at −80°C. We recommend saving 5 ml of the 3rd round dialysis buffer, to be stored in 1 ml aliquots, for subsequent dilutions of the protein and to serve as a negative control.

Biotinylated LacR Concentration • Timing 1 d

59. Approximate the concentration of the dialyzed LacR using a Nanodrop assuming an extinction coefficient of 28,545 ($M^{-1}cm^{-1}$) at 280 nm for the tagged protein. Blank the instrument using the 3rd round dialysis buffer (step 58).

60. Determine the concentration of the dialyzed LacR alongside a BSA standard curve using SDS-PAGE. Load between 400 – 25 ng of dialyzed LacR based on the Nanodrop reading from step 59.

61. Using the protein concentrations determined in steps 59–60, concentrate the LacR to approximately 750 ng/μl using an Amicon Ultra Centrifugal Filter unit, 10 kDa MWCO, at 14,000 × *g* in a microcentrifuge cooled to 4°C. Visually inspect the tube after every 10–30 minutes of centrifugation to determine the volume. Continue centrifugation until the volume is low enough that the expected protein concentration is 750 ng/μl (based on the nanodrop reading in step 59).

62. Repeat step 60 to determine the final concentration of LacR after concentration.

63. Flash freeze and store LacR at −80°C until ready to use.

Purification of DNA • *Timing 3 d*

CRITICAL Preparation of DNA should be performed independently of LacR purification

Chemical transformation of plasmid DNA for experimental approaches • Timing 2 h

64. Before transformation, complete the following:

    - Thaw the desired plasmids for transformation in room temperature water. Table 2lists the potential plasmids for transformation.
    - Warm LB/AMP and/or LB/AMP/IPTG agar plates (for plasmids containing *lacO* arrays) to 37°C.
    - Warm a water bath to 42°C.

65. Thaw 50 μl of chemically competent DH5α cells on ice and mix gently by pipetting up and down.

    CRITICAL STEP Do not subclone plasmids containing *lacO* arrays in strains harboring the $lacI^{Q}$ allele. This increases the concentration of cellular LacR and causes increased instability of any plasmids that harbor a *lacO* array.

66. Add 0.5 μl of each desired plasmid (~20 ng/μl) individually to the bottom of separate 15 ml falcon culture tubes and place each tube on ice. Add 0.5 μl of Tris-HCl (10 mM, pH 8.0) to a clean 15 ml falcon culture tube to serve as a negative control.

67. Add 50 μl of chemically competent DH5α cells to each tube containing the plasmids or the buffer. Mix by carefully swirling the tip of the pipet through the mix.

68. Incubate the transformation mixes on ice for 30 min.

69. Heat-shock the plasmid-cell mixture by incubating in a 42°C water bath for 45 sec.

70. Immediately place the tubes back on ice and incubate for 2 min.

71. Add 500 µl of room temperature SOC medium into each tube.

CRITICAL STEP If the plasmids contain *lacO* arrays, add IPTG to at least 2 mM to promote stable propagation of the plasmid.

72. Let the cells recover by incubating them in a 37°C shaking incubator set at 250 rpm for 60 min.

73. After the recovery period (step 72), mix the cells gently by pipetting and perform a 10-fold dilution using SOC medium.

74. Spread 100 µl of undiluted cells on one half of the plate and 100 µl of 10-fold diluted cells on the other half of the plate.

75. Incubate the plates for 16 h at 37°C or 30°C for plasmids containing *lacO* arrays.

CRITICAL STEP Growing plasmids containing *lacO* arrays at 30°C significantly increases plasmid stability.

CRITICAL STEP Do not grow plasmids containing *lacO* arrays for longer than 16 h as this will promote rearrangements of the plasmids.

76. After the 16 h incubation (step 75), remove the plates from the incubators and store at 4°C.

PAUSE POINT Store LB agar plates at 4°C for up to 2 days.

Amplification and isolation of plasmid DNA • Timing 20 h

77. Before preparing overnight cultures, perform the following:

- Warm LB agar plates from step 76 to room temperature.
- Prepare a mix of LB, AMP, and IPTG (if plasmid contains *lacO* arrays) for the desired number of overnight cultures as described below.

Component	Volume/Final Concentration
LB medium	4 ml
AMP (50 mg/ml)	100 µg/ml
IPTG (1M) (if plasmid contains *lacO* array)	2 mM

78. Add 4 ml of LB/AMP or LB/AMP/IPTG mix to 15 ml falcon culture tubes.

CRITICAL STEP For propagation of *lacO* array plasmids, do not grow cultures in larger volumes than 4 ml. This small culture volume limits rearrangements by minimizing population doublings. Midi/maxi preps are not recommended.

79. Using a P10 pipet tip, pick single at least 8 colonies from the plate from step 76 and inoculate them into 4 ml media (step 78).

CRITICAL STEP it is important to pick multiple colonies so that any subclones that undergo rearrangements in the *lacO* array can be screened out.

80. Incubate the cultures for 16 h at 37°C in a shaking incubator set to 250 rpm. Although growth on solid media plates at 30°C seems to be crucial for the stability of plasmids containing *lacO* arrays (step 75), this is much less of an issue in liquid media. Therefore, all liquid cultures are grown at 37°C.

81. After the 16 h incubation, remove the cultures from 37°C and chill on ice.

82. Use forceps to remove the P10 pipet tip (step 79) from each culture and discard into bleach.

83. Centrifuge the tubes at 2,500 RCF, 4°C for 5 min to pellet the bacteria.

84. Remove and discard the supernatant into bleach and invert the tube briefly on a paper towel to completely drain the pellet.

85. Store the pellets on ice until ready to miniprep.

PAUSE POINT Pellets can be frozen and stored at −20°C for several years.

86. Purify the plasmids using a QIAprep spin miniprep kit by following the manufacturer's instructions.

87. Elute the plasmid by adding 50 μl of Tris-HCl (10 mM, pH 8.0) into each miniprep column.

88. Measure the DNA concentration of each plasmid using a Nanodrop.

89. Store plasmids at −20°C.

Plasmid validation by restriction enzyme digestion • Timing 6 h

90. Check the *lacO* array sizes of the plasmid(s) by restriction digestion (Fig 4A). In a PCR tube, prepare a SacI/KpnI digestion mix on ice as described in the table below. Scale up the total digestion volume as needed. Perform single digestions as controls to confirm each enzyme cuts efficiently.

	SacI digestion	***Kpn*I digestion**	***Sac*I/*Kpn*I digestion**
Plasmid DNA (~100 ng/μl, step 89)	1	1	1
10X rCutsmart Buffer	1	1	1
*Sac*I-HF	0.2	-	0.2
*Kpn*I-HF	-	0.2	0.2
H_2O	7.8	7.8	7.6

91. Mix the digestion reaction by pipetting and incubate the tubes at 37°C for 1 h.

PAUSE POINT Incubate digestions at 37°C overnight.

92. During the incubation period (step 91), prepare an agarose gel (1% in TBE) and a 1 kb ladder.

93. Add 2 μl of DNA loading dye (6X, +SDS) to each digestion (step 91).

94. Load 6 μl of each digestion to each well of the gel alongside a 1 kb ladder. Store the remaining 6 μl of each digestion at 4°C in case the gel needs to be re-run.

95. Perform electrophoresis at 5 V/cm for approximately 2.5 h.

96. After electrophoresis, stain the gel using SYBR gold according to the manufacturer's instructions. Image the gel using a UV transilluminator.

97. Analyze the *lacO* array sizes. Select plasmids that contain the correct *lacO* array size for phenol/chloroform purification (Fig 4B).

TROUBLEHSOOTING

Phenol/chloroform purification of plasmid DNA • Timing 4 h

CRITICAL This protocol describes the purification of a single DNA template, but it can be scaled up as needed and multiple templates can be purified in parallel.

98. Before phenol/chloroform purification, prepare the following:

- Thaw plasmids validated by restriction enzyme digestion (steps 90–97).
- Chill ethanol (100% v/v and 70% v/v) on ice.
- Prepare an aliquot (220 μl per extraction) of phenol/chloroform/isoamyl alcohol (25:24:1) and allow to warm to room temperature.

  CRITICAL STEP Warm phenol/chloroform/isoamyl alcohol (25:24:1) to room temperature to prevent SDS precipitation from carrying over in subsequent steps.
- Prepare an aliquot (200 μl per extraction) of chloroform/isoamyl alcohol (24:1).
- Prepare Extraction Stop buffer.
- Cool a microcentrifuge to 4°C.

99. Pool 2–3 validated plasmids together up to 110 μl in a 0.5 ml Eppendorf safe-lock microcentrifuge tube. Note the concentration and volume used of each miniprep and use this information to calculate the total amount of input DNA, which is crucial for the subsequent resuspension step.

CRITICAL STEP If the volume of the pooled plasmids is less than 110 μl, dilute to 110 μl with Tris-HCl (10 mM pH 8.0). Volumes of below 110 μl will result in contamination of the DNA with either phenol/chloroform or chloroform, which will interfere with biochemical applications of the DNA.

100. Add 110 μl of Extraction Stop buffer to bring the total volume to 220 μl.

101. Mix by inversion and centrifuge briefly at 13,000 × *g* to collect all the liquid at the bottom of the tube.

102. Add 220 μl phenol/chloroform/isoamyl alcohol (25:24:1). Mix thoroughly by inverting the tube 10–15 times and then centrifuge at 13,000 × *g* for 4 min.

CRITICAL STEP This protocol has been empirically determined using 0.5 ml Eppendorf safe-lock microcentrifuge tubes to leave sufficient aqueous phase at each step so that the interface between aqueous and organic phases is not disrupted. This ensures that the DNA is not contaminated by phenol. Using different tubes may alter the volume of the aqueous phase that can be recovered without disturbing the interface.

103. Transfer 200 μl of the aqueous (upper) phase to a new 0.5 ml Eppendorf safe-lock microcentrifuge tube containing 200 μl chloroform/isoamyl alcohol (24:1). Mix thoroughly by inverting the tube 10–15 times and then centrifuge at 13,000 × *g* for 4 min.

104. Transfer 200 μl of the aqueous (upper) phase to a new 0.6 ml Axygen microcentrifuge tube containing 16 μl sodium acetate (3 M). Pipet up and down three times to mix.

105. Add 400 μl of pre-chilled 100% ethanol, then mix by gently inverting the microcentrifuge tube 5–10 times.

PAUSE POINT Samples in ethanol (100%) can be stored overnight at 4°C.

106. Incubate on ice for 15 min.

107. Centrifuge at 20,817 RCF, 4°C for at least 30 min. Prolonged centrifugation (up to 60 min) will modestly increase yield.

108. Remove all tubes from the centrifuge and keep them on ice. Use a vacuum line to aspirate all but approximately 50 μl of supernatant. To aspirate, hold the tube vertically to ensure the DNA pellet is not displaced. Aspirate down the opposite side of the DNA pellet. The DNA pellet may appear as a small white stripe towards the bottom of the tube.

109. Centrifuge at 20,817 RCF, 4°C for 1 min to bring all residual droplets to the bottom of the tube.

110. Use an ultrafine tip to carefully aspirate most of the remaining ethanol from the DNA pellet. Immediately add 400 μl of pre-chilled 70% ethanol.

CRITICAL STEP Use of an ultrafine tip is critical to not disturb the DNA pellet.

111. Centrifuge at 20,817 RCF, 4°C for 20 min.

112. Aspirate all but approximately 50 μl of supernatant again, as described in step 108.

113. Centrifuge at 20,817 RCF, 4°C for 1 min to bring all residual droplets to the bottom of the tube.

114. Use an ultrafine tip to carefully aspirate most of the remaining ethanol from the DNA pellet. Leave no more than 1–2 μl of ethanol behind. Cap the microcentrifuge tube and store it on ice until ready to resuspend the DNA.

CRITICAL STEP Use of an ultrafine tip is critical to not disturb the DNA pellet.

115. Uncap the microcentrifuge tube then transfer to a room temperature rack and let the residual ethanol evaporate (no more than 5 min).

116. Once the remaining ethanol has evaporated and the pellet has turned translucent, wait an additional 4 min before adding Tris-HCl (10 mM, pH 8.0) to resuspend the pellet at ~500 ng/μl.

CRITICAL STEP To determine how much Tris-HCl (10 mM, pH 8.0) is needed for resuspension of the pellet at 500 ng/μl, calculate the total amount of input DNA (step 99) and assume approximately 30% was lost during the purification procedure. We typically aim for a final DNA concentration of 300 ng/μl but resuspend pellets at 500 ng/μl to ensure the final DNA concentration is not below 300 ng/μl.

CRITICAL STEP It is important to not over- or under-dry the DNA pellet. We find that waiting 4 minutes after the pellet turns translucent balances these considerations.

117. Resuspend pellets by incubation at room temperature for no less than 30 min. Flick the tube and centrifuge briefly after 15 minutes and then once again at the end of the incubation.

118. (Optional) Incubate the DNA at 4°C overnight. This can improve DNA recovery if there are any concerns the DNA pellet was dried for too long (step 116).

119. Measure the concentration of the purified DNA using a Nanodrop, then add Tris-HCl (10 mM, pH 8.0) to adjust the concentration to 300 ng/μl for downstream applications.

120. Aliquot the DNA and store at −80°C to avoid freeze-thaw cycles of small volumes of DNA.

PAUSE POINT DNA can be stored at −80°C for several years

Preparation of modified DNA plasmids • *Timing 3 d*

CRITICAL In order to accept an oligonucleotide containing a DNA lesions, plasmid DNA must be nicked and purified, as described below.

Plasmid Nicking Preparation • Timing 3.5 h

121. Digest 20 μg of pJD194 or pJD195 (steps 64–120, Table 2) with Nb.*Bsm*I nicking enzyme in a 1.7 ml tube as detailed in the table:

Component	Volume (μl)
20 μg of pJD194 or pJD195 (300 ng/μl)	66.67
NEBuffer r3.1 (10X)	25
Nb.*Bsm*I	10
Nuclease-free H_2O	Up to 250

122. Mix the reaction by pipetting up and down and briefly centrifuge at 14,000 × *g* in a microcentrifuge to collect. Incubate the nicking reaction for 1 h at 37°C.

123. After 1 h, add extra nicking enzyme to ensure complete nicking of the plasmid DNA as described below:

CRITICAL STEP The final volume of the enzyme should not exceed 8% of the final reaction volume to prevent star activity.

Component	Volume (μl)
Nicking reaction (step 121)	250
NEBuffer r3.1 (10X)	5
Nb.*Bsm*I	14
Nuclease-free H_2O	31

CRITICAL STEP The final volume of the enzyme should not exceed 8% of the final reaction volume to prevent star activity.

124. Pipet up and down to mix and centrifuge in a microcentrifuge to collect. Incubate the nicking reaction for 1 h at 37°C.

125. Treat the samples with 5 μl of Proteinase K (10 mg/ml) for 1 h at 37°C to remove nicking enzymes. Pipet up and down to mix and briefly centrifuge at 14,000 × *g* in a microcentrifuge to collect.

126. Purify the nicking reaction using the Monarch® PCR & DNA Cleanup Kit and the Monarch® Plasmid Miniprep Columns according to the manufacturer's instructions. Elute into 30 μl and then transfer a 1 μl aliquot to a new tube and store at −20°C until quality control analysis is performed (steps 143–147).

CRITICAL STEP Monarch® Plasmid Miniprep columns have a binding capacity of 20 μg. Be sure not to exceed 20 μg per column, which will result in loss of nicked DNA.

PAUSE POINT Nicked DNA can be stored at −20°C for several years.

TROUBLEHSOOTING

Oligonucleotide Annealing and Ligation • Timing 19 h

CRITICAL This step is essential to introduce a DNA oligonucleotide containing a DNA lesion into a nicked plasmid substrate.

127. In a 1.7 ml microcentrifuge tube, anneal the oligo to the nicked DNA (from step 126) using a 100-fold molar excess of oligonucleotide, as described below. Scale up or down the volume of nicked DNA and oligonucleotide to account for differences in concentration.

Component	Volume (µl)
Nicked DNA (667 ng/µl) (step 126)	29
Oligonucleotide (100 µM, 24 bp)	7
Nuclease-free H_2O	Up to 50

128. Add the reaction tube to a heat block filled with water and set to 70°C. Incubate for 5 min.

CRITICAL STEP Be sure to not heat the DNA past 70°C, as this can cause plasmids to anneal to each other due to excessive denaturation.

129. Remove the tubes from the heat block and place them on a small stack of paper towels. Allow to slowly cool to room temperature (typically 1.5 – 2 h).

130. Once the annealing reaction has reached room temperature, assemble the ligation reaction in the same 1.7 ml tube, as follows:

Component	Volume (µl)
Annealed DNA (step 127)	50
rCutsmart (10X)	7
ATP (0.2 M)	2
T4 DNA ligase	4
Nuclease-free H_2O	Up to 70

TROUBLEHSOOTING

131. Incubate the ligation reaction at room temperature overnight in the dark. Once the ligation is completed, transfer a 1 µl aliquot to a new tube and store at −20°C until quality control analysis is performed (steps 143–147).

PAUSE POINT Ligated DNA can be stored at −20°C for several years.

Enzymatic Cleanup • Timing 6 h

CRITICAL Here, we describe how to perform the enzymatic removal of re-annealed template DNA or unligated products of annealing. We have found this enzymatic approach to be more effective than gel extraction or electroelution-based approaches.

132. Treat the ligated DNA with *Age*I-HF enzyme as described below. For quality control, perform a scaled-down *Age*I-HF digestion using input DNA (the same prep of pJD194 or pJD195 used in step 121) to confirm successful digestion.

Component	Volume (µl)
Ligation reaction (step 130)	70
rCutsmart (10X)	13
*Age*I-HF	10
Nuclease-free H_2O	Up to 200

TROUBLEHSOOTING

133. Pipet up and down to mix and centrifuge briefly to collect to the bottom of the tube. Incubate for 1 h at 37°C then store a 4 µl aliquot at −20°C until quality control analysis is performed (steps 143–147).

134. To remove nicked (unligated) and linear (reannealed) plasmids, digest the DNA with T5 Exonuclease (2 U/µg of plasmid DNA) as follows:

Component	Volume (µl)
*Age*I digestion reaction (step 132)	200
rCutsmart (10X)	3
T5 exonuclease	4
Nuclease-free H_2O	Up to 250

TROUBLEHSOOTING

135. Pipet up and down to mix and centrifuge in a microcentrifuge to collect. Incubate the T5 Exonuclease digestion reaction for 3 h at 37°C then store a 4.6 µl aliquot at −20°C until quality control analysis is performed (steps 143–147).

136. Equilibrate the membrane of an Amicon Ultra-0.5 Centrifugal Filter Unit with 100 kDa MWCO. Add 400 µl of Tris-HCl (10 mM, pH 8.0) to the column, place the column in the provided collection tube, and centrifuge at 14,000 × *g* for 2 min. Discard the flow through.

137. Add the T5 exonuclease digested DNA (from step 134) to the column, then add 150 µl Tris-HCl (10 mM, pH 8.0) to a final volume of 400 µl. Centrifuge at 14,000 × *g* for 2 min. Discard the flow through.

138. Add 400 µl of Tris-HCl (10 mM, pH 8.0) to the column. Centrifuge at 14,000 × *g* for 2 min. Discard the flow through. Repeat this step two more times.

139. To recover the DNA, place the column upside down in a fresh collection tube. Centrifuge at 14,000 × *g* for 2 min to collect. This will result in approximately 35 µl of buffer exchanged DNA.

140. After buffer exchange, transfer a 1 µl aliquot to a new tube and store at −20°C until quality control analysis is performed (steps 143–147).

141. Measure the concentration of the purified DNA (A260/A280) using a Nanodrop.

142. Store at −20°C until ready to use.

PAUSE POINT DNA can be stored at −20°C for several years.

Quality control • Timing 4 h

143. Dilute quality control samples (steps 126, 131, 133, 135, and 140 to 10 µl with Tris-HCl (10 mM, pH 8.0) and then add 2 µl DNA loading dye (6X, +SDS).

144. Prepare the following samples for analysis (as in step 143):

- A 1 kb ladder aliquot
- An aliquot of input pJD194 or pJD195 (ideally this is the same aliquot of pJD194 or pJD195 used in step 121)

145. Prepare a 1% (w/v) agarose gel in 1x TBE buffer and add ethidium bromide (0.3 mg/ml) (Fig 5B).

146. Load the samples from steps 143 and 144 and run the gel at 5 V/cm for at least 90 min or until the dye front reaches halfway down the gel.

147. Image the gel using a UV transilluminator. Ligation reactions should result in a decrease in nicked DNA and an increase in supercoiled DNA. *Age*I digest should result in a small increase in additional species, T5 Exonuclease should remove essentially all species other than the supercoiled DNA, and the supercoiled DNA should be fully *Age*I resistant (Fig 5B).

PROCEDURE 2: Experimental approaches • Timing 1–2 d

In vitro DNA replication using LacR as a reversible replication barrier • Timing 4–8 h

CRITICAL This protocol is for a single reaction but should be scaled up or down as needed.

CRITICAL We recommend Brandtech pipettors and pipet tips for maximum accuracy and reproducibility.

1. Combine 1 µl of *lacO* array plasmid (Table 2) (300 ng/µl) from procedure 1: step 120, 2 µl of LacR (750 ng/µl) from procedure 1: step 63, and 0.4 µl of pJD145 (loading control plasmid) from procedure 1: step 120 (Fig 6A). Set up a control reaction lacking LacR, using an equal volume of LacR dialysis buffer instead. Mix gently by pipetting up and down 5–8 times with BrandTech P20 tips. (Optional) In order to study replication of damaged DNA, plasmid DNA templates harboring DNA damage (from procedure 1: step 142) can be substituted for *lacO* array plasmid.

2. Incubate at room temperature for at least 1.5 h to allow LacR to bind DNA.

3. During the LacR binding period (step 2), distribute 40 µl of Extraction Stop buffer into microcentrifuge tubes, one for each sample.

4. Thaw a 33 µl aliquot of HSS, then add 1 µl of ARS and 0.2 µl Nocodazole (0.5mg/ml). Mix by pipetting up and down 20–30 times.

5. Centrifuge HSS at 13,000 × *g* for 5 min at room temperature to remove any insoluble debris. This 5-minute incubation step also allows time for the HSS to activate.

6. Transfer 30 μl of activated HSS into a new tube and avoid transferring any pellet that formed during step 5. Carefully mix the activated HSS by pipetting up and down 8–10 times.

7. After incubation of LacR with DNA (step 2), add 4.25 μl of activated HSS to the 0.75 μl LacR-*lacO* array DNA mix to initiate licensing ('licensing mix'). Mix thoroughly by pipetting up and down 10–20 times. Incubate for 30 min at room temperature.

   CRITICAL STEP Avoid bubbles while mixing the licensing mix and move the tip around the tube while pipetting to mix thoroughly.

8. Approximately 10 min after the start of step 7, prepare an aliquot of NPE mix (45.45%) by thawing a 20 μl aliquot of NPE and adding 0.6 μl of ARS, 0.858 μl of DTT (50 mM), 4.8 μl of ELB (5X), 1 μl of [α-^{32}P] dATP, and 16.7 μl of H_2O . Mix by pipetting up and down 20–30 times. Incubate for at least 15 min at room temperature.

   CAUTION When handling radioactive material for this step and all subsequent steps wear proper personal protective equipment (PPE), work behind a shield, and follow all local radiation safety procedures.

   CRITICAL STEP Do not scale up [α-^{32}P] dATP volume, as this can introduce variability.

9. After incubation with HSS for 30 min (step 7), initiate replication by mixing 2 volumes of NPE mix (step 8) with 1 volume of licensing mix (step 7). Mix thoroughly by pipetting up and down 10–20 times. The minimum recommended reaction size is 10 μl.

   CRITICAL STEP Licensing mix and NPE mix should each comprise 1/3 of the final reaction. Typically, this is achieved by diluting the NPE to a 50% mix and mixing 2:1 with licensing mix. If small molecule inhibitors need to be added to the reaction (e.g. aphidicolin in step 10), then NPE should be less dilute to account for the additional volume.

   CRITICAL STEP When initiating multiple reactions, stagger initiation by at least 1 min.

   TROUBLESHOOTING

10. (Optional) Steps 1–9 are required for all replication reactions involving a LacR barrier. Follow option A to subsequently induce site-specific uncoupling of replication forks (Fig 2A, 2B lane 5), or option B to induce termination of replication forks (Fig 2A, 2B lane 4), then subsequently perform steps 11–12:

    a. Site-specific uncoupling of the replication forks: prepare aphidicolin release mix immediately after initiating replication (step 9). Add 10%

(v/v) of the final reaction volume (step 12) of Aphidicolin release mix to the bottom of new microcentrifuge tubes for each reaction.

b. Termination of replication forks: prepare IPTG (250 mM in ELB (1X)) immediately after initiating replication (step 9). Add 4% (v/v) of the final reaction volume (step 12) of IPTG (250 mM in ELB (1X)) to the bottom of new microcentrifuge tubes for each reaction.

11. (Optional) Prior to induction of uncoupling or termination, sample the reaction at the desired time points before LacR release by adding 2 μl of the reaction to 40 μl of Extraction Stop buffer. Pipet up and down rapidly to ensure the full 2 μl of reaction is added to the Extraction Stop buffer. Flick the tube to ensure thorough mixing. This step can be performed to assess replication forks prior to LacR release.

CRITICAL STEP When sampling multiple reactions, stagger sampling as per initiation (step 9).

TROUBLEHSOOTING

12. (Optional) At the desired LacR release time, follow option A to release LacR and induce termination (Fig 2A, 2B lane 4), or option B to induce site-specific uncoupling of replication forks (Fig 2A, 2B lane 5). The omission of these steps keeps the LacR barrier intact to maintain replication fork stalling (Fig 2A V, 2B lanes 3 & 6).

A. *Release of LacR and termination:* add the reaction mix to 4% final reaction volume of IPTG (to a final concentration of 10 mM). Mix thoroughly by pipetting up and down 10–20 times.

B. *Site-specific uncoupling of the replication forks:* add the reaction mix to 10% final reaction volume of Aphidicolin release mix (Aphidicolin + IPTG) (Fig 6a). Mix thoroughly by pipetting up and down 10–20 times.

CRITICAL STEP When releasing multiple reactions, stagger release as per initiation (step 9).

TROUBLEHSOOTING

13. Sample the reaction at desired time points as in step 11.

14. Add 1.9 μl of RNAse (2 mg/ml) to all samples and incubate at 37°C for at least 30 min or overnight at room temperature.

15. Add 1.83 μl of Proteinase K (20 mg/ml) to all samples and incubate at 37°C for at least 30 min or overnight at room temperature.

PAUSE POINT RNAse and Proteinase K treated samples can be stored at 4°C for up to 1 week. Store at −20°C for up to 4 weeks.

Separation of replication intermediates using native agarose gels • Timing 4 h

16. Transfer 10 μl of each sample (step 15) to a tube then add 2 μl of DNA loading dye (6X - SDS). Mix thoroughly. Note that SDS is not required in the loading dye because it is present in the extraction stop (steps 13 and 11).

    PAUSE POINT Samples in DNA loading dye can be stored at 4°C for up to 1 week or at −20°C for up to a month.

17. Prepare a 120 ml (16 cm × 14 cm gel tray) or 220 ml (20 cm × 20 cm gel tray) agarose gel (1% (w/v) in TBE (1X)).

18. Load approximately 1.5 μl of each sample per 1 mm of lane width.

19. Perform electrophoresis at 5 V/cm for approximately 2.5 h or until the dye front has migrated ~10 cm.

20. After performing electrophoresis, cut the gel ~1 cm above the dye front. Discard the bottom half of the gel in a radioactive waste container as this contains most of the unincorporated radionucleotides.

21. Place the gel face down on plastic wrap. Cover the gel with a piece of 0.2 μm nitrocellulose and 2 pieces of Whatman paper cut to the same size as the agarose gel.

22. Place a ~5” stack of paper towels on top of the gel and apply a weight of ~1 kg for at least 1 h to compress the gel.

23. **PAUSE POINT** Gels can compress overnight or up to 2 days.

24. Remove the paper towels and the top Whatman paper, and then carefully flip the gel and replace the top Whatman paper, now in direct contact with the gel. Place the gel in this same orientation, with the nitrocellulose facing down, in a gel drier heated to 80°C attached to a vacuum pump.

25. After the gel is fully dried, remove the bottom Whatman paper and wrap the gel in plastic wrap. When the gel has fully dried in a gel drier, the bottom Whatman paper (the one contacting the membrane) will curl up and detach from the membrane.

26. Visualize the gel by phosphorimaging and analyze the gel using ImageJ (see Procedure 4).

    PAUSE POINT Dried gels can be stored indefinitely but the signal will decrease ~2 fold every ~14 days due to the half life of ^{32}P.

    TROUBLESHOOTING

PROCEDURE 3: Analytical gels • Timing 4 d

Phenol/chloroform purification of DNA replication intermediates • Timing 6 h

1. Before phenol/chloroform purification, prepare the following:

    - Warm RNAse and Proteinase K treated DNA samples from replication reactions (procedure 2: step 15) to room temperature if stored at 4°C.

- Chill ethanol (100% v/v and 70% v/v) on ice.
- Prepare an aliquot (220 µl per extraction) of phenol/chloroform/isoamyl alcohol (25:24:1) and allow to warm to room temperature.

  CRITICAL STEP Warm phenol/chloroform/isoamyl alcohol (25:24:1) to room temperature to prevent SDS precipitation from carrying over in subsequent steps.
- Prepare an aliquot (200 µl per extraction) of chloroform/isoamyl alcohol (24:1).
- Prepare Extraction Stop buffer.
- Cool a microcentrifuge to 4°C.

2. To each DNA sample (procedure 2: step 15) add Extraction Stop buffer to bring the volume to 110 µl total.

   CRITICAL STEP Volumes of below 110 µl will result in contamination of the DNA with either phenol/chloroform or chloroform, which will interfere with enzymatic treatment of the purified DNA.

3. Add 110 µl of Tris-HCl (10 mM, pH 8.0) to bring the total volume to 220 µl.

4. Mix by inversion and centrifuge briefly at 13,000 × *g* to collect all the liquid at the bottom of the tube.

5. Add 220 µl phenol/chloroform/isoamyl alcohol (25:24:1). Mix thoroughly by inverting the tube 10–15 times and then centrifuge at 13,000 × *g* for 4 min.

   CRITICAL STEP This protocol has been empirically determined using 0.5 ml Eppendorf safe-lock microcentrifuge tubes to leave sufficient aqueous phase at each step so that the interface between aqueous and organic phases is not disrupted. This ensures that the DNA is not contaminated by phenol. Using different tubes may alter the volume of the aqueous phase that can be recovered without disturbing the interface.

6. Transfer 200 µl of the aqueous (upper) phase to a new 0.5 ml Eppendorf safe-lock microcentrifuge tube containing 200 µl chloroform/isoamyl alcohol (24:1). Mix thoroughly by inverting the tube 10–15 times and then centrifuge at 13,000 × *g* for 4 min.

7. Prepare new tubes containing 1 µl glycogen (20 mg/ml) and 16 µl sodium acetate (3 M).

   CRITICAL STEP Make a master mix of sodium acetate (3M) and glycogen and mix by vigorously pipetting up and down 5–10 times before vortexing for 30–60 sec. The glycogen promotes efficient precipitation of low quantities of DNA and allows the pellet to be easily visualized. Improper mixing of the glycogen-sodium acetate master mix will result in variable glycogen addition and inconsistent recovery of DNA.

8. Transfer 200 µl of the aqueous (upper) phase (step 6) to a new tube containing glycogen and sodium acetate (step 7). Mix by pipetting up and down 5 times.

9. Add 400 µl of pre-chilled 100% ethanol, then mix by gently inverting the microcentrifuge tube 5–10 times.

   PAUSE POINT Samples in ethanol (100%) can be stored overnight at 4°C.

10. Incubate on ice for 15 min.

11. Centrifuge at 20,817 RCF, 4°C for at least 30 min. Prolonged centrifugation (up to 60 min) will modestly increase yield.

12. Remove all tubes from the centrifuge and keep them on ice. Use a vacuum line to aspirate all but approximately 50 µl of supernatant. To aspirate, hold the tube vertically to ensure the DNA pellet is not displaced. Aspirate down the opposite side of the DNA pellet. The DNA pellet may appear as a small white stripe towards the bottom of the tube.

13. Centrifuge at 20,817 RCF, 4°C for 1 min to bring all residual droplets to the bottom of the tube.

14. Use an ultrafine tip to carefully aspirate most of the remaining ethanol from the pellet, which will be white and visible due to the glycogen. Immediately add 400 µl of pre-chilled 70% ethanol.

    CRITICAL STEP Use of an ultrafine tip is critical to not disturb the DNA pellet.

15. Centrifuge at 20,817 RCF, 4°C for 20 min.

16. Aspirate all but approximately 50 µl of supernatant again, as described in step 12.

17. Centrifuge at 20,817 RCF, 4°C for 1 min to bring all residual droplets to the bottom of the tube.

18. Use an ultrafine tip to carefully aspirate most of the remaining ethanol from the DNA pellet. Leave no more than 1–2 µl of ethanol behind. Cap the microcentrifuge tube and store it on ice until ready to resuspend the DNA.

    CRITICAL STEP Use of an ultrafine tip is critical to not disturb the DNA pellet.

19. Uncap the microcentrifuge tube then transfer to a room temperature rack and let the residual ethanol evaporate (no more than 5 min).

20. Once the remaining ethanol has evaporated and the pellet has turned translucent, wait an additional 4 min before adding Tris-HCl (10 mM, pH 8.0) to resuspend the pellet at approximately 5 ng/µl. To determine the resuspension volume, calculate the amount of DNA sampled during *in vitro* DNA replication and assume 70% recovery efficiency. We typically sample 2 µl during replication and resuspend DNA in 3 µl Tris-HCl (10 mM, pH 8.0).

CRITICAL STEP It is important to not over- or under-dry the DNA pellet. We find that waiting 4 minutes after the pellet turns translucent balances these considerations.

21. Resuspend pellets by incubation at room temperature for no less than 30 min. Flick the tube and centrifuge briefly after 15 minutes and then once again at the end of the incubation.

22. (Optional) Incubate the DNA at 4°C overnight. This can improve DNA recovery if there are any concerns the DNA pellet was dried for too long (step 20).

23. Store extracted DNA samples at 4°C for up to 1 week or at −20°C for up to one month.

TROUBLESHOOTING

Separation of replication fork structures using native agarose gels • Timing 5 h

24. Digest 1 μl of the purified replication intermediates (step 23). Use option A to analyze pairs of replication forks when the DNA structure corresponding to two converging replication forks is well established (e.g., two canonical replication forks, which form a ‘double Y’ structure). Use option B to analyze single replication forks when the DNA structure corresponding to two converging replication forks is complex or not well established (e.g., if one of the forks undergoes replication fork reversal).

A. To analyze pairs of replication forks: Digest the purified replication intermediates with one unit of *Xmn*I enzyme in rCutSmart Buffer (1X) with a reaction volume of 10 μl (Fig 7A). Perform digestion for 1 hour at 37°C. Note that any restriction enzyme that cuts only once and is located away from the *lacO* array can be used in the place of *Xmn*I.

Component	Amount
Purified Replication intermediates (step 23)	1 μl
rCutSmart Buffer	1 μl
*Xmn*I (20,000 units/ml)	0.05 μl
Water (up to 10 μl)	

B. To analyze individual replication forks: Digest the purified replication intermediates with one unit each of XhoI and DraIII-HF enzymes, in rCutSmart Buffer (1X) with a reaction volume of 10 μl (Fig 8D–G). Perform digestion for 1 hour at 37°C.

Component	Amount
Purified Replication intermediates (step 23)	1 μl

Component	Amount
rCutSmart Buffer	1 µl
*Dra*III-HF (20,000 units/ml)	0.05 µl
*Xho*I (20,000 units/ml)	0.05 µl
Water (up to 10 µl)	

TROUBLESHOOTING

25. Incubate the reaction at 37°C for 1 h.

26. Add 2 µl of DNA loading dye (6X, +SDS) to 10 µl of digestion reaction. Flick the tubes to mix then briefly centrifuge at 14,000 × *g*.

PAUSE POINT Store *Xmn*I digested samples at 4°C for up to 1 week. Store at −20°C for up to 4 weeks.

27. Repat procedure 2: steps 16–25 to separate the replication fork structures using native agarose gels (Fig 7B–C).

Separation of replication fork structures using 2D native agarose gels • Timing 2 d

28. Prepare a solution of 220 ml SeaKem GTG agarose (0.4% w/v) and freshly made TBE buffer (1X) by microwaving until all agarose is melted. Note: SeaKem agarose is used for 2D native gels to ensure high quality.

CRITICAL STEP TBE (10X) should always be made fresh before using for 2D gels.

29. Stir constantly until the mixture cools to ~55°C.

30. Place 1 mm well combs on both ends of a 20 cm × 20 cm casting tray and push both well combs to one side of the casting tray in the same orientation so the wells are aligned.

31. Once the mixture from step 29 is cooled to ~55°C, pour the mixture into the casting tray.

32. After the gel has solidified, submerge the gel in an electrophoresis tank containing TBE buffer (1X).

33. Allow the gel to set for at least 30 min before performing 1D electrophoresis.

CRITICAL STEP Best results are typically achieved if the gel sets for 1 h – 1.5 h before electrophoresis.

34. Load approximately 1.5 µl of 1 kb ladder and *Xmn*I digested sample or *Xho*I/*Dra*II digested sample (from step 24) per 1 mm lane of lane width. Load samples in lanes flanked on both sides by empty wells.

CRITICAL STEP Samples are loaded in lanes flanked on both sides by empty wells so 1D gel samples are easily cut out as individual gel slices for the 2D gel (step 38).

35. Perform 1D electrophoresis at 1 V/cm for 21 h.

36. After 1D electrophoresis is complete, use an X-acto knife to cut out the gel slice containing the 1 kb ladder. To ensure accurate cutting, align a flexible ruler down the edge of one gel slice, using the wells at the top and bottom of the gel as a guide. Perform the cutting in the casting tray. The gel slice should contain 3 lanes, the 1 kb ladder flanked by two blank lanes. Any alternative approach that allows the gel to be accurately cut will also suffice, but we find this approach works best for most investigators.

37. Soak the gel slice in TBE (1X) and ethidium bromide (0.5 μg/ml) while shaking gently for 15 min to stain the 1 kb ladder.

38. While the 1 kb ladder is staining, use an X-acto knife to cut out gel slices containing each sample, as described in step 36. The gel slices should contain 3 lanes each, the sample flanked by two blank lanes. Discard any gel slices that do not contain samples.

39. After staining, image the 1 kb ladder gel slice alongside a fluorescent ruler in a UV transilluminator.

CRITICAL STEP Only image the 1 kb ladder, but not the remaining lanes to avoid damage to the DNA.

40. Using the 1 kb ladder image from the UV transilluminator, cut each gel slice containing the samples (step 38) to include ~1 – 2.5X the appropriate molecular weight range, plus ~1 cm each side.

CRITICAL STEP Cut gel slices that span from 1.5 kb to 12 kb, typically ~10 cm.

41. Set up a 20 cm × 20 cm gel casting tray with one 2 mm well comb and seal the ends with shipping tape. Note that any size casting tray will work if the voltage is adjusted appropriately (step 50).

CRITICAL STEP Ensure the casting tray is large enough to fit at least 1 gel slice.

42. Place each gel slice parallel to the comb and carefully push the gel slice up against the teeth of the comb so it is straight. Maneuver the gel slices one at a time onto a flexible ruler and carefully slide each gel slice off the ruler on to the casting tray.

43. Prepare a solution of 220 ml SeaKem GTG agarose (1.2% w/v) and TBE buffer (1X) by microwaving until all agarose is melted.

CRITICAL STEP The amounts provided are sufficient for a 20 cm × 20 cm gel tray. Scale gel volume for larger or smaller gel trays, as appropriate.

44. Stir constantly until the mixture cools to ~55°C.

CRITICAL STEP The agarose must be cooled to ~55°C before pouring the 2D gel, otherwise the heat may disrupt fragile replication intermediates present in the gel slice.

45. Once the temperature is ~55°C, add ethidium bromide (0.3 μg/ml) and stir for an additional minute.

46. Carefully pour the dissolved agarose and ethidium bromide into the casting tray then immediately remove the comb. The dissolved agarose and ethidium bromide does not need to cover the entire gel slice but does need to cover at least the bottom edge of the gel slice so DNA from the 1D gel slice can enter the 2D gel.

CRITICAL STEP Use a pipet tip to dislodge any bubbles that form around the gel slice as these will block DNA from entering the gel.

47. Allow the 2D gel to cool and solidify for at least 30 min.

48. After the gel has cooled and solidified, submerge the gel in an electrophoresis tank containing TBE buffer (1X) and ethidium bromide (3 μg/ml).

49. Place the electrophoresis tank at 4°C and let incubate for at least 30 min.

50. Run the gel at 5 V/cm 4°C, for 12 h.

PAUSE POINT After the gel has finished running, it can be stored at 4°C overnight.

51. Place the gel face down on plastic wrap. Cover the gel with a piece of Hybond N+ and 2 pieces of Whatman paper cut to the exact size of the agarose gel.

52. Place a ~5” stack of paper towels on top of the gel and apply weight of ~1 kg for >2 h to compress the gel.

PAUSE POINT Gels can compress overnight or up to 2 days.

53. Remove the paper towels and the top Whatman paper, and then carefully flip the gel and replace the top Whatman paper, now in direct contact with the gel. Place the gel in this same orientation, with the Hybond N+ facing down, in a gel drier heated to 80°C attached to a vacuum pump.

54. After the gel is fully dried, remove the bottom Whatman paper and wrap the gel in plastic wrap. Note that when the gel has fully dried in a gel drier, the bottom Whatman paper (the one contacting the membrane) will curl up and detach from the membrane.

55. Visualize the gel by phosphorimaging and analyze the gel using ImageJ (see Procedure 4).

PAUSE POINT Dried gels can be stored indefinitely but the signal will decrease ~2 fold every ~14 days due to the half life of ^{32}P.

Separation of nascent strands using denaturing agarose gels • Timing 1 d

56. Prepare a digestion of 1 μl purified replication intermediates (step 23) with one unit of *Alw*NI enzyme in rCutSmart Buffer (1X) with a reaction volume of 10 μl. *Alw*NI is used instead of *Xmn*I (in step 24), because *Alw*NI digest yields leftward nascent strands (LWS) and rightward nascent strands (RWS) of greater size difference (Fig 9A). Using *Xmn*I at this point would make it

more challenging to distinguish the LWS and RWS as their migration during electrophoresis would be more similar.

Component	Amount
Purified Replication intermediates (Step 23)	1 µl
rCutSmart Buffer	1 µl
*Alw*NI (10,000 units/ml)	0.1 µl
Water (up to 10 µl)	

TROUBLESHOOTING

57. Incubate the reaction at 37°C for 1 h.

58. Add 1 µl EDTA (330 mM) to the 10 µl digestion reaction. Flick the tubes to mix then briefly centrifuge.

CRITICAL STEP EDTA is added to chelate all the Mg^{2+} which would otherwise precipitate out in the alkaline sample/migration buffer and disrupt migration of DNA out of the wells. For this reason, it is critical to add EDTA before adding alkaline loading buffer (containing NaOH).

59. Add 2.2 µl alkaline loading buffer (6X, +NaOH) to each sample. Flick the tubes to mix then briefly centrifuge.

PAUSE POINT Store *Alw*NI digested samples at 4°C for up to 1 week. Store at −20°C for up to 4 weeks.

60. Prepare a solution of 108 ml (16 cm × 14 cm gel tray) or 198 ml (20 cm × 20 cm gel tray) SeaKem GTG agarose (1.67% w/v) and water by microwaving thoroughly until all the agarose is melted.

61. Stir constantly until the mixture cools.

62. Once the gel has cooled to ~55°C, add $1/9^{th}$ volumes of alkaline buffer (10X) to yield an agarose gel (1.5% w/v) in alkaline buffer (1X). After addition of the alkaline buffer, the final gel volume should be of 120 ml or 220 ml, respectively.

CRITICAL STEP Make alkaline buffer (10X) fresh before each use.

CRITICAL STEP Let the gel cool before addition of alkaline buffer (10X). If alkaline buffer (10X) is added before the gel is cooled, the gel will not solidify.

63. Continue stirring the gel for another minute, then pour the gel into the casting tray.

64. After the gel has cooled and solidified, submerge the denaturing gel in an electrophoresis tank containing alkaline buffer (1X).

CRITICAL STEP Submerge the denaturing gel in alkaline buffer (1X) for at least 1h before electrophoresis to ensure the gel is fully set.

65. Load approximately 1.5 µl of each sample per 1 mm lane of lane width.

66. Perform electrophoresis at 1.5 V/cm for approximately 18 h or until the dye front has migrated ~15 cm. Note that much of the dye will diffuse out of the gel during long periods of electrophoresis. To increase the visibility of the dye front, load undiluted loading dye in one of the outer lanes or put a glass plate on top of the gel.

67. After electrophoresis, rinse the gel with water then transfer to a new container and cover with water.

68. Add sufficient TCA (7% (w/v)) to neutralize the gel and incubate for 30 min with gentle agitation. The volume of TCA (7% (w/v)) typically required is ~10% the volume of the gel (step 62) but this will vary depending on the exact preparation of running buffer and the amount of evaporation that has taken place during the electrophoresis (step 66). It is therefore recommended that the required amount of TCA be empirically determined. To do this, perform the following steps:

- Remove a small volume of gel running buffer (from step 66) after the run (typically 4 ml) and add TCA (7% (w/v)) to this buffer sample until the pH reaches 6.5, as determined using pH strips.
- Scale up the amount of TCA (7% (w/v)) according to the volume of the gel. For example, if 0.4 ml of TCA (7% (w/v)) is needed to neutralize 4 ml of running buffer (as is typical) then 12 ml of TCA (7% (w/v)) is needed for a 120 ml gel and 22 ml is needed for a 220 ml gel.

CRITICAL STEP If the gel is not neutralized, then the structure of the agarose gel will be disrupted when heat is applied during drying (steps 72 and 74), which will limit the ability to detect nascent DNA strands.

69. Once the gel is neutralized, trim off the top (wells and above) and bottom of gel to ensure it lays flat.

70. Place the gel face down on plastic wrap. Cover the gel with a piece of Hybond N+ and 2 pieces of Whatman paper cut to the exact size of the agarose gel.

71. Place a ~5” stack of paper towels on top of the gel and apply weight of ~1 kg for >2 h to compress the gel.

PAUSE POINT Gels can compress overnight or up to 2 days.

72. Remove the paper towels and the top Whatman paper, and then carefully flip the gel and replace the top Whatman paper, now in direct contact with the gel. Place the gel in this same orientation, with the Hybond N+ facing down, in a gel drier heated to 60°C attached to a vacuum pump.

73. (Optional) Before the gel is almost dried, wrap it in plastic wrap and visualize by phosphorimaging (procedure 4: steps 1–7). This step can be performed if the gel degrades when dried with heat (steps 72 and 74). However, performing this step can however increase the risk of contamination of the phosphorimaging screen and exposure cassette.

74. Once the gel is almost dry, increase the temperature of the gel drier to 80°C to completely dry the gel.

    CRITICAL STEP Denaturing gels are sensitive to high heat and can only tolerate 80°C for short amounts of time (~10–15 min). Therefore, they should only be subjected to high heat once they are almost completely dry.

75. After the gel is fully dried, remove the bottom Whatman paper and wrap the gel in plastic wrap. Note when the gel has fully dried in a gel drier, the bottom Whatman paper (the one contacting the membrane) will curl up and detach from the membrane.

76. Visualize the denaturing gel by phosphorimaging and analyze the gel using ImageJ (Procedure 4).

    PAUSE POINT Dried gels can be stored indefinitely but the signal will decrease ~2 fold every ~14 days due to the half life of ^{32}P.

    TROUBLESHOOTING

PROCEDURE 4: Data acquisition and analysis • Timing 2 h – 4 d

CRITICAL Users should ideally have access to a phosphorimager so that replication intermediates can be analyzed by phosphorimaging. In the absence of a phosphorimager, X-ray film could also be used but the resulting data may be less quantitative owing to the reduced dynamic range of the film. For this reason, we only discuss imaging of replication intermediates by phosphorimaging.

Phosphorimaging • Timing 1 h – 4 d

1. Before phosphorimaging, blank a phosphorimager screen on a light box for at least 10 min.

2. Place the dried gel in plastic wrap (procedure 2: step 24 and procedure 3: steps 54 & 75) in an exposure cassette with the membrane facing upwards.

    CRITICAL STEP Ensure the membrane is facing upwards. If the membrane is facing downwards, gel images may be out of focus because the membrane is further from the screen.

3. Place the phosphorimager screen phosphor (white) side down on top of the gel.

4. Close the cassette and expose the gel for at least 30 min, up to 96 hours. Note that exposure time depends on the radiation date of [α-^{32}P] dATP, how much [α-^{32}P] dATP was used, and the type of gel. Denaturing and 2D gels typically require longer exposure times.

    TROUBLESHOOTING

5. Record the final exposure time and scan the gel using a phosphorimager with background subtraction and 100 μM resolution (Fig 2B, 6B, 7B, 8B, 8F, 9B).

6. After the gel has finished scanning (step 5), save it as a TIF file and record the maximum intensity. If the maximum intensity is <10,000, re-expose the gel (steps 1–5) and scale the re-exposure time as follows: Re-exposure time = [Max intensity] / [50,000] × original exposure time.

7. After scanning, blank the phosphorimager screen on a light box after for at least 20 min.

ImageJ analysis • Timing 30 min

8. Open the TIF file from step 6 in ImageJ.

9. Use the Window/Level tool (Image → Adjust → Window/Level) to maximize visibility of signal on the gel. To do this, first narrow the visual window (we typically use a value of ~5,000), then increase the level until all bands of interest are clear and background is visible.

10. After the Window/Level is adjusted appropriately, close the Window/Level panel (click the 'X' button within the panel) but do not click "Apply".

    CRITICAL STEP Do not click "Apply" after adjusting the Window/Level as this alters the underlying data rather than just the view.

11. Rotate the image so that all gel lanes are straight.

12. Measure the whole lane signal of each lane.

13. Measure the signal of the bands of interest.

14. Measure the background signal of each lane. Typically, we measure the background signal for whole lanes immediately above the well. For individual bands on native gels, we typically measure the background immediately below the band of interest to avoid any signal that smears up the lane due to variability in migration. For individual bands on denaturing gels, we measure the background immediately above the band of interest because the signal generally smears downwards due to the formation of transient secondary structures, even under denaturing conditions.

15. Copy and paste the resulting measurements from steps 12–14 into a spreadsheet software.

16. Subtract the background mean signal from each whole lane or band of interest mean signal.

17. Calculate the signal by multiplying the results from step 16 by the area value of each whole lane or band of interest.

18. If a loading control was included, normalize signal to the loading control.

19. Plot the signal as arbitrary units (AU), % of whole lane, and/or as % of replication intermediates at T=0 (Fig 6C, 7C, 8C, 8G, 9C).

TROUBLESHOOTING

Troubleshooting advice can be found in table 3.

TIMING

Procedure 1: Preparation of materials, 10.5 d

Steps 1–63 Purification of lac repressor (LacR), 4.5 d

- Steps 1–14, Chemical transformation of plasmid containing LacR, 2 h
- Steps 15–20, Biotinylated LacR Expression, 20 h
- Steps 21–53, Biotinylated LacR Purification, 1 d
- Steps 54–58, Biotinylated LacR Dialysis, 20 h
- Steps 59–63, Biotinylated LacR Concentration, 1 d

Steps 64–120, Purification of DNA, 3 d

- Steps 64–76, Chemical transformation of plasmid DNA for experimental approaches, 2 h
- Steps 77–89, Amplification and isolation of plasmid DNA, 20 h
- Steps 90–97, Plasmid validation by restriction enzyme digestion, 6 h
- Steps 98–120, Phenol/chloroform purification of plasmid DNA, 4 h

Steps 121–147, Preparation of modified DNA plasmids, 3 d

- Steps 121–126, Plasmid Nicking Preparation, 3.5 h
- Steps 127–131, Oligonucleotide Annealing and Ligation, 19 h
- Steps 132–142, Enzymatic Cleanup, 6 h
- Steps 143–147, Quality control, 4 h

Procedure 2: Experimental approaches, 1–2 d

Steps 1–15, In vitro DNA replication using LacR as a reversible replication barrier, 4–8 h

Steps 16–25, Separation of replication intermediates using native agarose gels, 4 h

Procedure 3: Analytical gels, 4 d

Step 1–23, Phenol/chloroform purification of DNA replication intermediates, 6 h

Step 24–27, Separation of replication fork structures using native agarose gels, 5 h

Steps 28–55, Separation of replication fork structures using 2D native agarose gels, 2 d

Steps 56–76, Separation of nascent strands using denaturing agarose gels, 1 d

Procedure 4: Data acquisition and analysis, 2 h-4 d

- Steps 1–7, Phosphorimaging, 1 h - 4 d
- Steps 8–19, ImageJ analysis, 30 min

ANTICIPATED RESULTS

This protocol efficiently produces LacR, DNA plasmids containing *lacO* arrays, and modified DNA plasmids containing site-specific DNA damage adjacent to *lacO* arrays. These reagents can be combined with *Xenopus* egg extracts to control replication fork movement and study various aspects of DNA replication and replication-coupled DNA repair.

LacR purification should be assessed by SDS-PAGE (Fig 3). Most of the protein is present in the insoluble fraction (Fig 3B, compare lanes 1 and 3). After sonication and addition of ammonium sulfate, the resulting precipitate is relatively pure (Fig 3B, lane 3). Binding to a monomeric avidin column and elution with biotin typically results in a single prominent band, indicating high purity (Fig 3B, lanes 8–10). The final overnight elution should yield relatively little protein (Fig 3B, lane 11), indicating that the prior elution steps were efficient.

lacO-containing plasmids are susceptible to rearrangements of the *lacO* sequences, presumably due to the difficulties associated with replicating repetitive sequences. Therefore, it is important to analyze each preparation of *lacO*-containing plasmid DNA after subcloning. To this end, we carry out restriction digests of individual DNA preparations to analyze the size of the *lacO* array-containing fragment by agarose gel electrophoresis (Fig 4). Most plasmid preparations do not undergo rearrangements (Fig 4B, lanes 6,9–12). However, some preparations will inevitably undergo rearrangements, resulting in increases or decreases in the length of the restriction fragment containing the *lacO* sequences (Fig 4B, lanes 5,7,8). Importantly, a single preparation can be heterogeneous, with each individual species being low abundance (Fig 4B, species indicated with arrows in lanes 5,8 compared to lane 6). For this reason, it is important to use a high-sensitivity detection approach. We favor the high sensitivity DNA dye SYBR gold (as outlined in this protocol) because it is well characterized and compatible with the standard UV transillumination equipment that is typically used to visualize DNA. Alternatively, DNA sequencing approaches can be used but these are much less economical and can take much longer if sequencing equipment is not readily available.

To engineer site-specific DNA damage into plasmid DNA, we introduce a modified oligonucleotide into a host plasmid (Fig 5A). This approach is efficient, as shown by the fact that most of the host plasmid ends up fully ligated and containing the modified oligonucleotide (Fig 5B, compare lanes 4 and 7). We do note that the procedure is inefficient between nicking and ligation because most of the nicked DNA is lost (Fig 5B, compare lanes 3 and 4). However, it is relatively easy to produce nicked plasmid DNA, so we have not optimized the procedure further. Importantly, after ligation, most of the host plasmid contains the modified oligonucleotide (Fig 5B, there is little change in the abundance of highest mobility species between lanes 4–5) and this cannot be attributed to incomplete

digestion by *Age*I, which cut to completion (Fig 5B, lane 8). Finally, the subsequent purification steps have a negligible effect on DNA abundance (compare lanes 5–7).

Under typical conditions, replication of plasmid DNA in *Xenopus* egg extracts results in supercoiled circular monomers (Fig 2A and Fig 2B lanes 1–2). Supercoiling arises because the plasmid DNA becomes chromatinized upon addition to extracts[61] and undergoes compensatory supercoiling when the reaction is stopped and the DNA is deproteinized. Replication in the presence of a LacR barrier results in a high molecular weight species corresponding to θ structures (Fig 2B, lane 3), which are molecules that are mostly replicated by a pair of replication forks (Fig 2A). These θ structures persist over time (Fig 2B, lane 6) due to the ability of the LacR barrier to block replication forks (Fig 2A). Importantly, addition of IPTG results in formation of the same supercoiled species that normally arise from completion of DNA synthesis (Fig 2B, compare lanes 2 and 4) due to disruption of the LacR barrier and termination of DNA replication (Fig 2A)[13]. These data show that the LacR barrier can block replication fork progression and is reversible (as shown in[13]). Additionally, the small molecule inhibitor aphidicolin can be added to inhibit DNA polymerases. When aphidicolin is added along with IPTG, the replication machinery continues to unwind in the absence of replicative helicase activity (Fig 2A)[24]. This results in ‘uncoupling’ of replication forks, which produces a unique high mobility θ* species (Fig 2B, lane 5) due to reannealing of the parental DNA strands and compensatory supercoiling upon deproteinization of the DNA (as in[24]). Replication fork uncoupling leads to nascent strand degradation and replication fork reversal (Figs 7–9), which can be analyzed using the analytical assays described in this protocol.

These analytical assays allow for a comprehensive analysis of nascent strand degradation and fork reversal (as in[24]). The upstream process of replication fork uncoupling can be assessed by analyzing unprocessed DNA species on a native agarose gel (Fig 6). Uncoupling results in formation of θ* species (Fig 6B) and loss of the original θ species (Fig 6C). To determine the extent of nascent strand degradation, we typically digest purified DNA intermediates with a restriction enzyme and then analyze by native agarose gel electrophoresis (Fig 7). Restriction digestion removes topological effects and allows replication fork structures to be clearly distinguished from linear products that are fully unwound (compare RIs in Fig 7B to θs in Fig 6B). Nascent strand degradation results in loss of signal from replication fork structures with no commensurate increase in the linear products (Fig 7C). As a more sensitive read-out for degradation, digested DNA can also be analyzed by denaturing gel electrophoresis to measure the onset of degradation (Fig 9). Degradation of nascent DNA strands reduces their size, which results in loss in signal the moment degradation begins (Fig 9B), allowing the onset of degradation to be measured (Fig 9C). Restriction digests of replication intermediates can also be analyzed by 2-D gel electrophoresis to determine the relative abundance of double Ys, which correspond to the structures ordinarily formed by pairs of replication forks, compared to reversed forks (Fig 8A–C). We typically observe a background of approximately 20% reversed forks but, upon induction of uncoupling, most forks undergo reversal within 60 minutes (Fig 8C). It can be challenging to unambiguously identify DNA structures involving multiple replication forks (Fig 8A). For this reason, it can be useful to perform 2-D gel electrophoresis of a single population of replication forks moving in a single direction by performing a restriction

digest to isolate replication forks localized to only one side of the LacR barrier (Fig 8D–E). This allows for reversed forks to be clearly observed and quantified (Fig 8F–G).

Although the assays described here are mostly used in the context of nascent strand degradation and fork reversal, they can also be used to study many aspects of DNA replication. Native DNA gels (Fig 6) can be used to identify a range of topoisomers formed during DNA replication[13] and replication-coupled DNA repair[19,62]. Native gel analysis of restriction digested DNA (Fig 7) allows unambiguous determination of whether replication forks remain on the DNA[13,27], which can act as a read-out of replication fork progression[21,23]. Denaturing gel analysis of digested DNA (Fig 9) allows us to unambiguously determine the size of nascent DNA strands, which can act as a read-out for replication fork position[13,27]. Denaturing gel analysis can also assess whether the daughter DNA strands are fully ligated[13,27]. 2-D gel electrophoresis (Fig 8) allows for a range of replication fork structures[13] and intermediates of replication-coupled DNA repair[19,63] to be resolved. Thus, the approaches described here can illuminate multiple aspects of DNA replication.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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DATA AVAILABILITY

Sequences for plasmid DNA templates (Table 2) are available upon request. Source data are provided with this paper.

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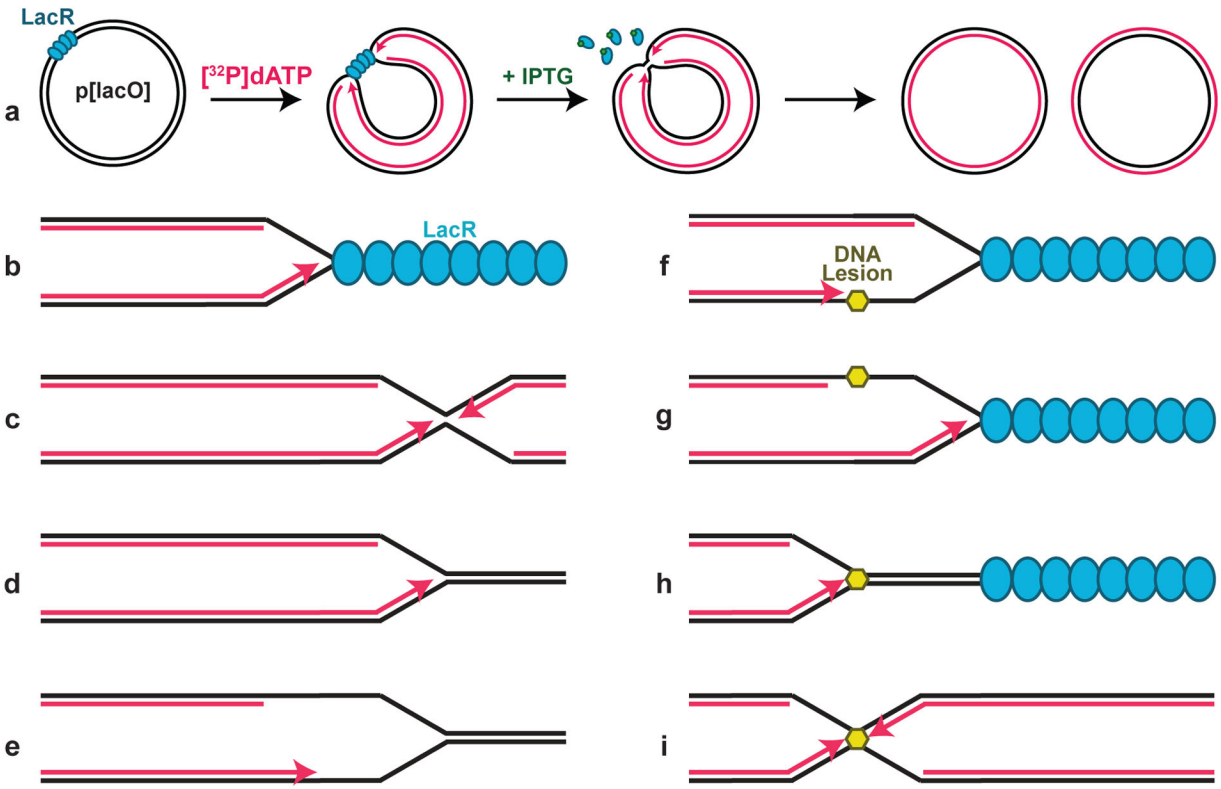

Fig 1. Control of replication fork movement by a lac repressor (LacR) array

a, Plasmid DNA harboring a *lac* operator array (p[*lacO*]) is incubated with LacR (in blue) to assemble a site-specific replication barrier ('LacR barrier') Regardless of when and where replication is initiated on the plasmid, replication forks ultimately end up arrested either side of the LacR barrier. Addition of IPTG disrupts the LacR barrier, which allows replication forks to restart and ultimately complete DNA synthesis. Nascent DNA strands can be radiolabeled by inclusion of radioactive dATP ([^{32}P]dATP) to allow replication intermediates and products to be detected. **b**, Schematic of a replication fork stalled at a LacR barrier. **c**, Schematic of two terminating forks converging after disruption of the LacR barrier. **d**, Schematic of an elongating fork after disruption of the LacR barrier. Analysis of fork progression *without* termination can be readily achieved by increasing the size of the LacR barrier to delay the encounter with a converging fork. **e**, Schematic of an 'uncoupled' replication fork after disruption of the LacR barrier and inhibition of DNA polymerases (e.g., using aphidicolin). 'Uncoupling' occurs when DNA polymerase activity is inhibited while activity of the replicative helicase is allowed to proceed. This results in unwinding of the parental DNA strands (black) without synthesis of daughter DNA strands (red). **f**, Schematic of a replication fork encountering a DNA lesion on the leading strand template. The LacR barrier blocks the replication fork moving in the opposite direction to ensure only a single fork encounters the lesion on a specific strand. **g**, Schematic of a replication fork encountering a DNA lesion on the leading strand template. Strand specific encounter by a single fork is enforced by the LacR barrier (as in (f)). **h**, Schematic of a single replication fork encountering a DNA lesion that involves both DNA strands. Encounter by a single fork is enforced by the LacR barrier (as in (f)). **i,** Schematic of two replication forks converging

upon a DNA lesion that involves both DNA strands. This can be enforced by omitting the LacR barrier or adding IPTG to disrupt it.

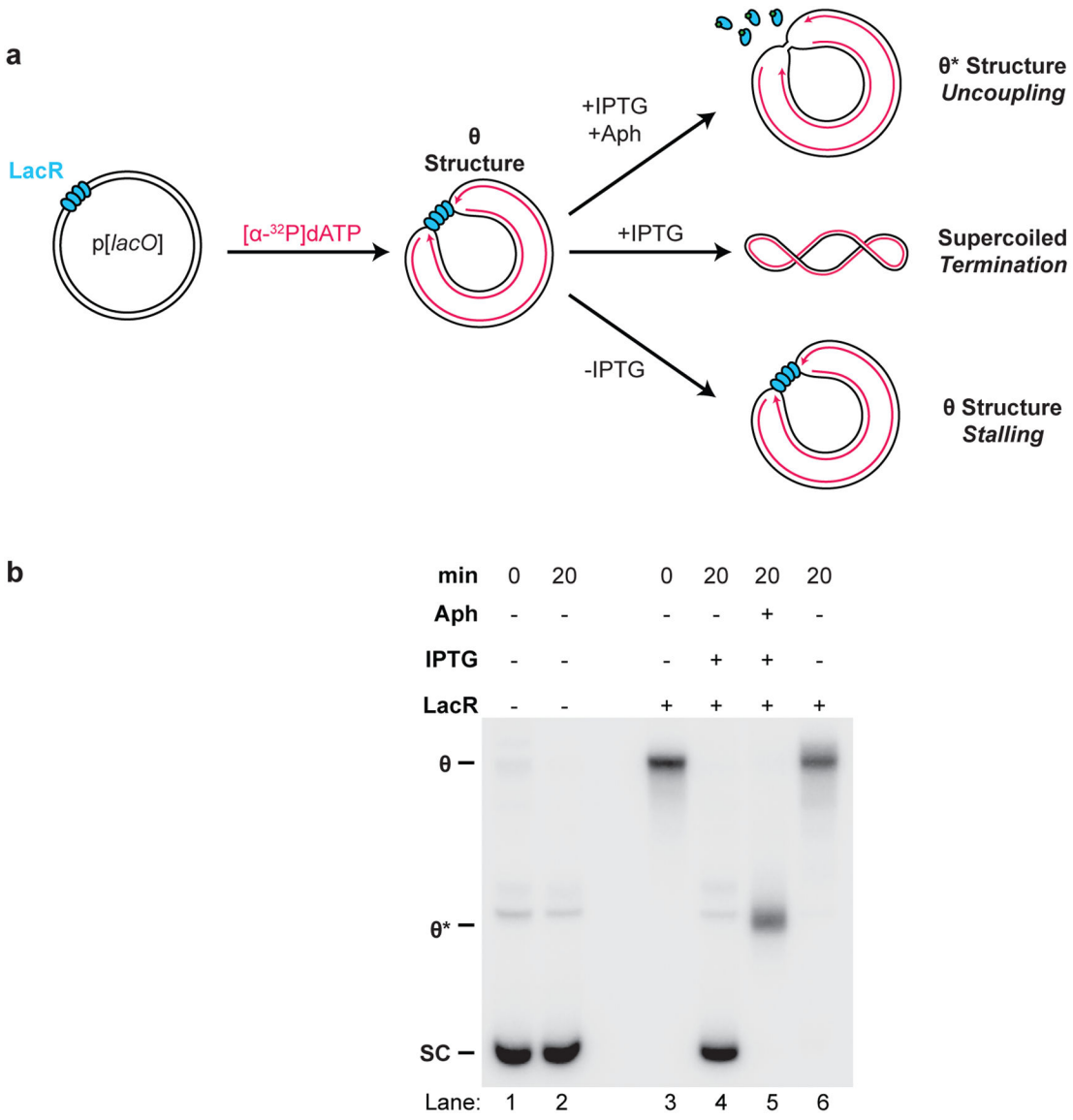

Fig 2. Use of a LacR barrier to control replication fork termination, uncoupling, and stalling.

a p[*lacO*] was replicated in *Xenopus* egg extracts in the presence of dATP[α-^{32}P] to label the nascent DNA strands. Once forks were stalled at the barrier, the reaction was split and treated with IPTG to induce termination, IPTG plus aphidicolin to induce uncoupling, or vehicle to maintain replication fork stalling.

b, Samples treated as described in **a** were separated on an agarose gel and visualized by autoradiography. Note that the dramatic change in mobility for the θ* species arises from compensatory supercoiling when the DNA is deproteinized and parental DNA strands reanneal (also see Fig 6A).

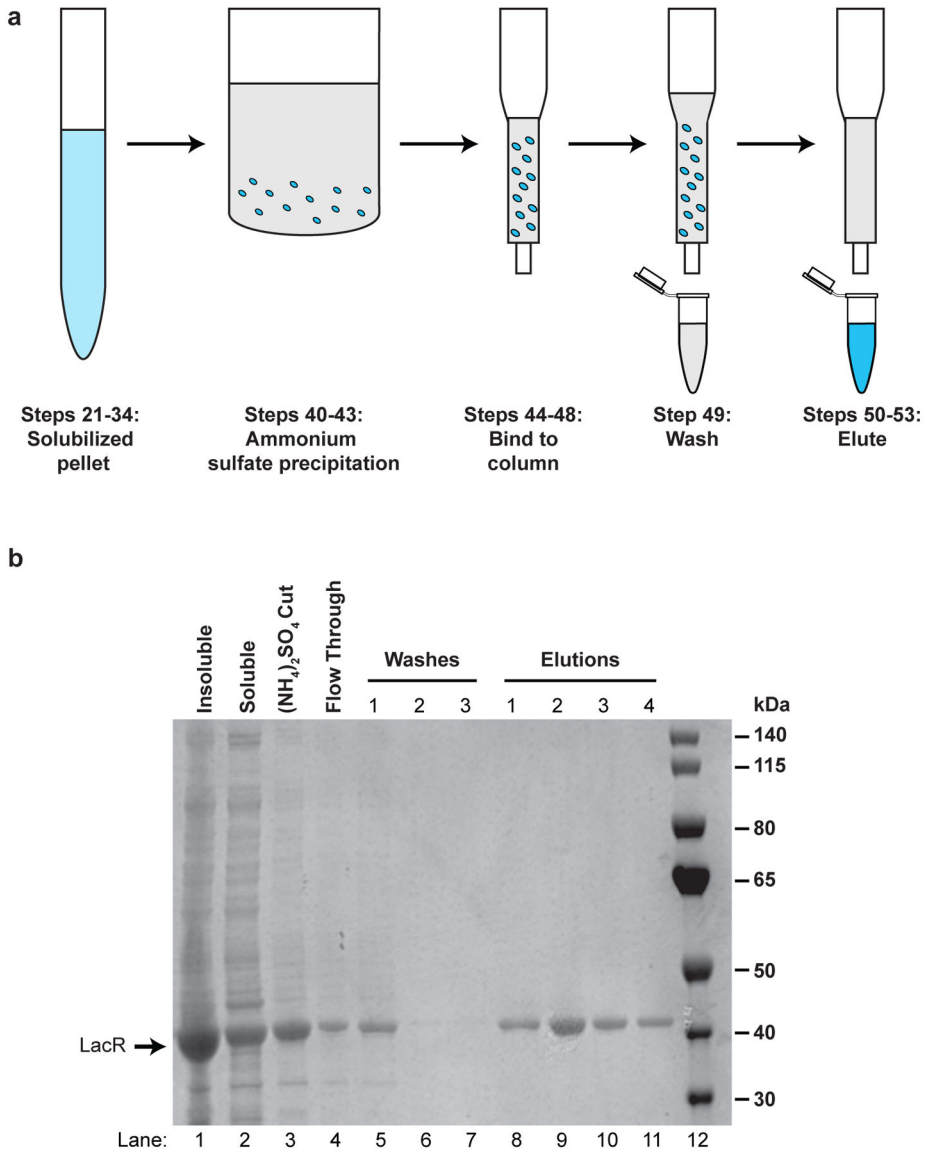

Fig 3. Purification of biotinylated LacR

a, Schematic of the key steps in the LacR purification procedure. First, a bacterial cell pellet containing biotinylated LacR is solubilized (Procedure 1: Steps 21–34). Next, the biotinylated LacR is precipitated with ammonium sulfate to remove most soluble proteins (Procedure 1: Steps 40–43). Biotinylated LacR is then resuspended and bound to a monomeric avidin column (Procedure 1: Steps 44–48). Non-specifically bound proteins are washed away (Procedure 1: Step 49) and, finally, biotinylated LacR is eluted by addition of biotin (Procedure 1: Steps 50–53). **b**, Samples from **a** were separated on an SDS-PAGE gel and visualized by Coomassie staining. Note that elution 4 (lane 11) was performed overnight.

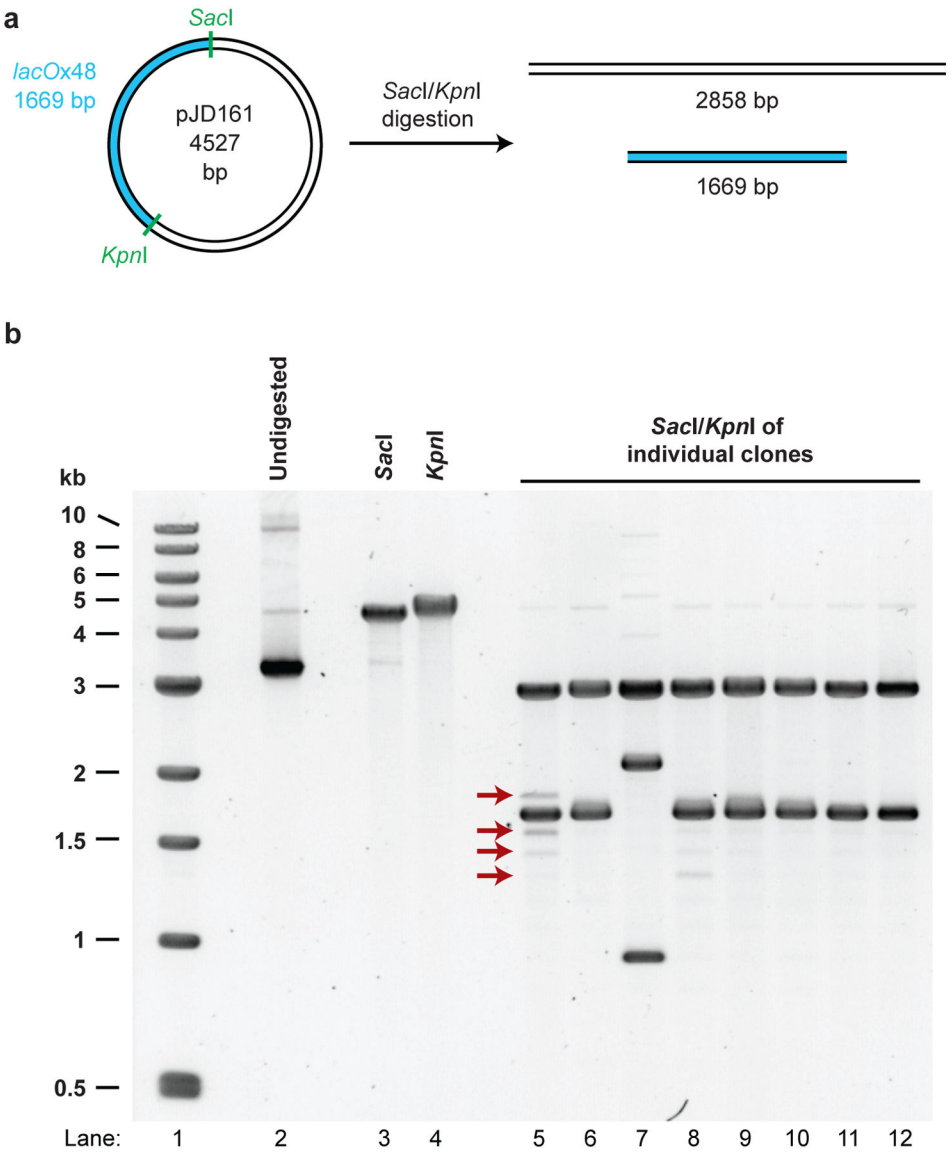

Fig 4. Quality control of subcloned lacO array plasmids

a, Cartoon depicting pJD161 and the expected fragment sizes after *Sac*I/*Kpn*I digestion. SacI and KpnI restriction sites on the plasmid are depicted in green, *lacO* array is depicted in blue. **b**, Eight subclones of pJD161 were restriction digested with *Sac*I and *Kpn*I, and separated on an agarose gel and detected by SYBR gold-staining (lanes 5–12). Undigested DNA (lane 2) and single digested DNA (lanes 3 and 4) were also included as a positive control for restriction enzyme activity (lanes 2–4). Note that clones in lanes 5, 7, 8 did not pass plasmid validation. Red arrows indicate altered *lacO* array sizes within a subset of molecules in subclones shown in lanes 5 and 8.

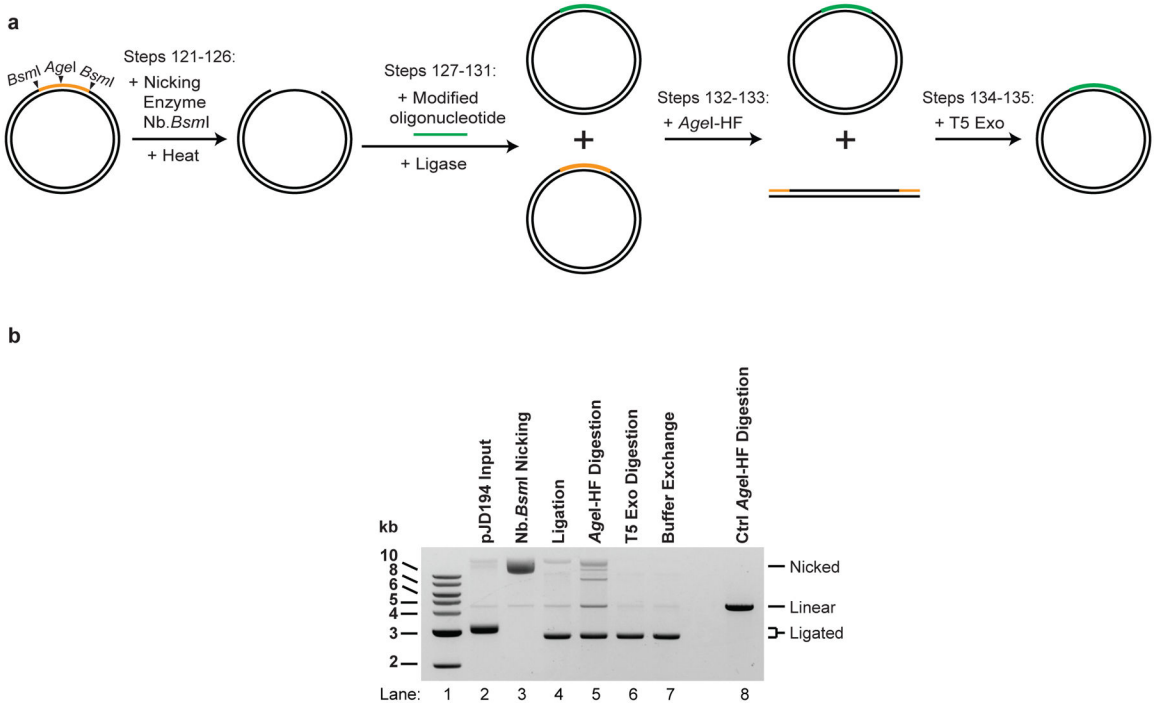

Fig 5. Preparation of plasmids containing modified nucleotides

a, Schematic of the procedure for preparing plasmid DNA containing modified nucleotides. First, a host plasmid is nicked with *Nb.Bsm*I either side of the sequence 5’ – CATTCACCGGTATCCTTACGAGCG – 3’ (orange) and the intervening DNA is melted off using heat (Procedure 1: Steps 121–126). Next, a modified oligonucleotide (green) is annealed into the gapped host plasmid and ligated (Procedure 1: Steps 127–131). The products of ligation include plasmids that contain the modified oligonucleotide (green) as well as those containing the parental DNA sequence (orange). To distinguish these, the modified oligonucleotide is engineered so that the DNA damage is within the *Age*I recognition site (ACCGGT). This allows the ligated products to be treated with *Age*I to linearize plasmids containing the parental DNA sequence, while leaving those containing the modified oligonucleotide untouched (Procedure 1: Steps 132–133). Treatment with T5 Exonuclease is then used to degrade the linearized molecules that contain the parental DNA (Procedure 1: Steps 134–135). Note that T5 Exonuclease also degrades any nicked DNA molecules arising from incomplete ligation. **b**, Samples from (a) were separated on an agarose gel and visualized by ethidium bromide staining. The modified oligonucleotide in this case (SDO2) contained a single nucleotide mismatch (5’ – CATTCACTGGTATCCTTACGAGCG – 3’) within the *Age*I recognition site (ACCGGT). Note that the parental plasmid (lane 2) is negatively supercoiled while the ligated products (lanes 4–7) are not. Thus, the parental plasmid (lane 2) undergoes less positive supercoiling in response to ethidium bromide compared to the ligated products (lanes 4–7).

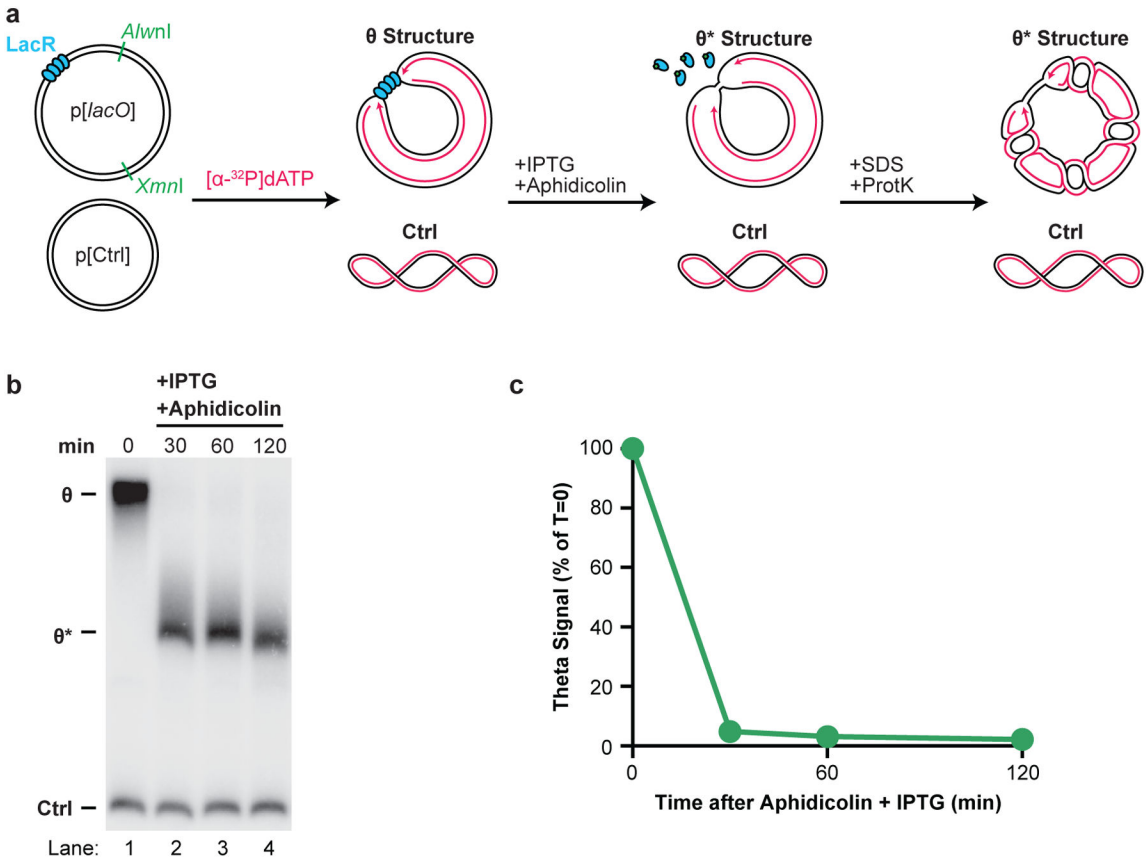

Fig 6. Replication fork uncoupling using a LacR barrier.

a, p[*lacO*] containing *Xmn*I and *Alw*nI restriction digest sites (in green) was replicated in *Xenopus* egg extracts in the presence of dATP[α-^{32}P] to label the nascent DNA strands. In parallel a control plasmid without a *lacO* array was replicated (p[Ctrl]). Once forks are stalled at the barrier, IPTG plus aphidicolin was added to induce uncoupling on p[*lacO*]. p[Ctrl] was unaffected by IPTG and aphidicolin addition. Uncoupling resulted in θ* structures where the parental DNA strands were unwound. After samples were withdrawn from the reaction they were deproteinized by addition of SDS and Proteinase K. For θ* structures this resulted in compensatory catenane formation due to reannealing of the parental DNA strands. **b**, Samples from **a** were separated on an agarose gel and visualized by autoradiography to visualize the different DNA intermediates of DNA replication. **c**, Quantification of *θ* structures, which correspond to replication fork structures, from **b**. Loss of θ structures is due to replication fork uncoupling, which generates θ* structures.

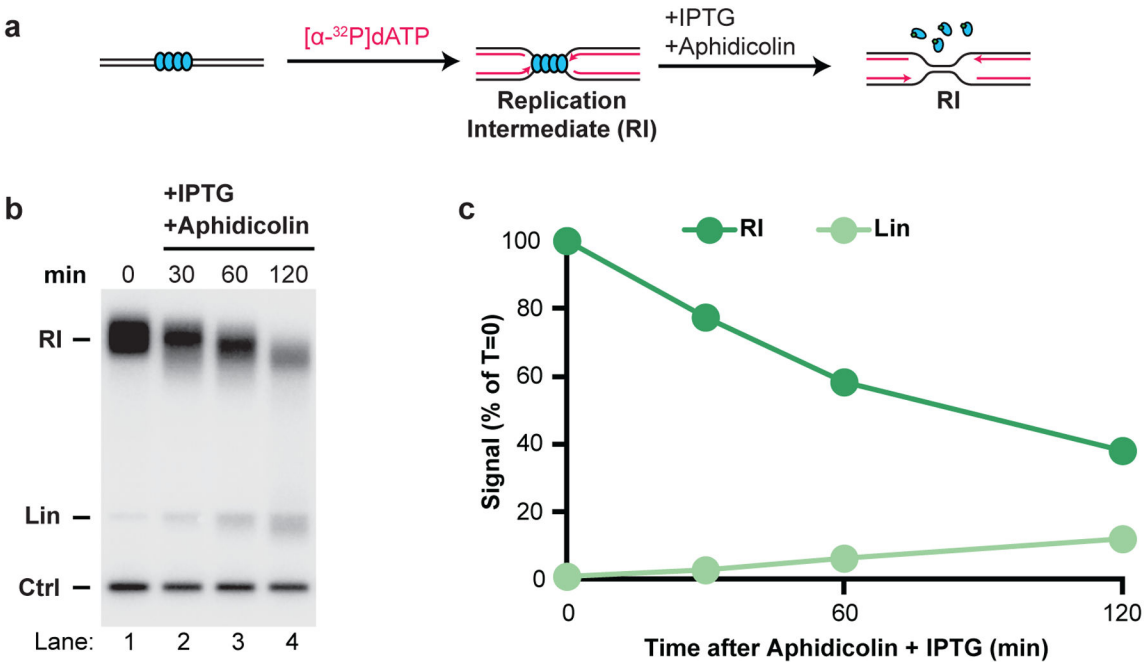

Fig 7. Native gel analysis of replication fork structures

a, The DNA intermediates of DNA replication from **6a** were purified and digested with *Xmn*I to identify replication intermediates (RI) and linear products (lin) of replication. **b**, Samples from **a** were separated on an agarose gel and visualized by autoradiography. RI species increase in mobility over time and few linear products of replication are produced, suggesting that degradation takes place. **c**, Quantification of RI and lin species from **b**. RI species decrease without a commensurate increase in the linear products of replication, which shows that the RI species are degraded.

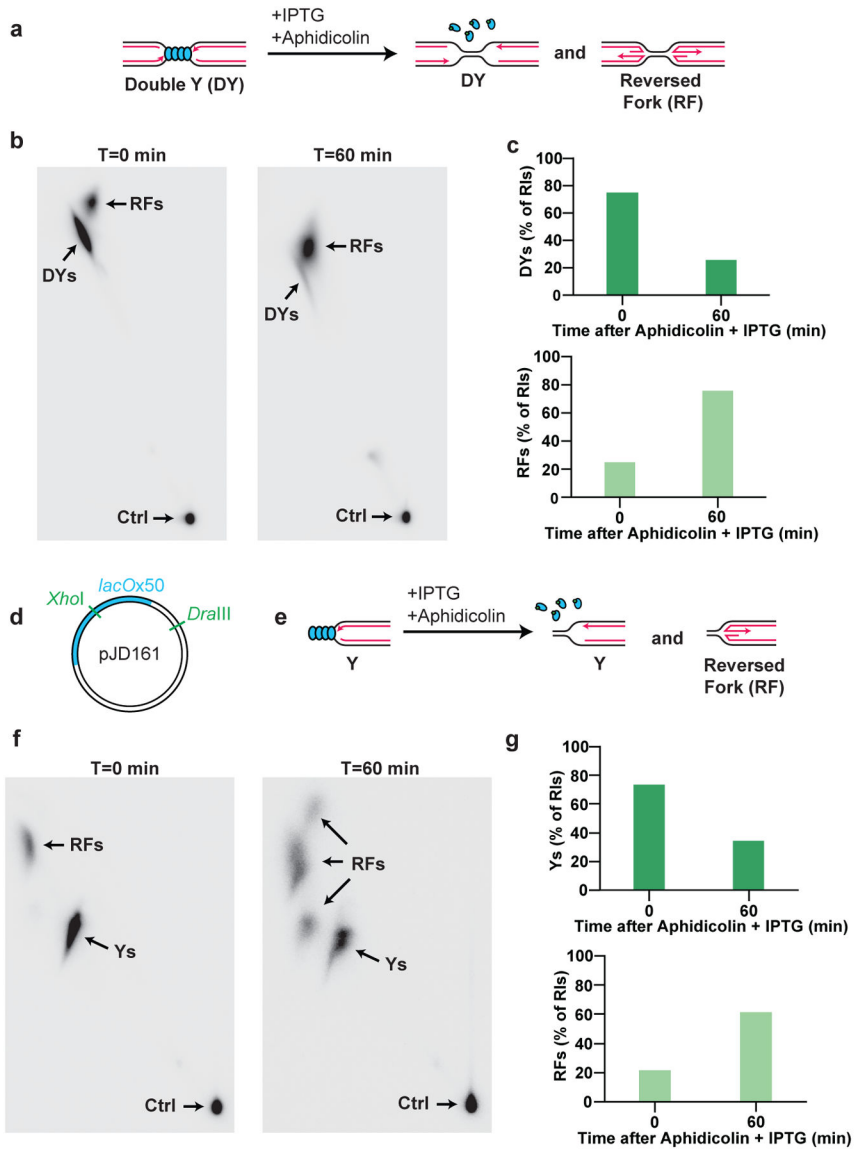

Fig 8. 2D gel analysis of replication fork structures

a, The DNA intermediates of DNA replication from **6a** were purified and digested with *Xmn*I to yield double Ys (DYs) and reversed forks (RFs). **b**, Samples from **a** were separated by 2D electrophoresis to and visualized by autoradiography. Prior to addition of IPTG and aphidicolin (0 min) most of the signal is present in DYs because replication fork structure is unaltered. After addition of IPTG and aphidicolin (60 min), uncoupling was induced, resulting in most of the signal shifting to RFs because fork reversal took place. **c**, Quantification of DYs and RFs from **b**. Between 0 and 60 min DYs decrease in abundance while RFs increase in abundance due to fork reversal. **d**, Cartoon indicating *Xho*I and *Dra*III restriction digest sites on pJD161 used to excise individual replication forks for 2D gel analysis. **e**, The DNA intermediates of DNA replication from **6a** were purified and digested with *Xho*I and *Dra*III to yield a single replication fork (Ys) and reversed forks (RFs). **f**, Samples from **e** were separated by 2D electrophoresis and visualized by autoradiography.

Prior to addition of IPTG and aphidicolin (0 min) most of the signal is present in Ys because replication fork structure is unaltered. After addition of IPTG and aphidicolin (60 min), uncoupling was induced, resulting in most of the signal shifting to RFs because fork reversal took place. Note that multiple RF species are present due to degradation by the DNA2 nuclease (as in[24]). **g**, Quantification of Ys and RFs from **f**. Between 0 and 60 min Ys decrease in abundance while RFs increase in abundance due to fork reversal.

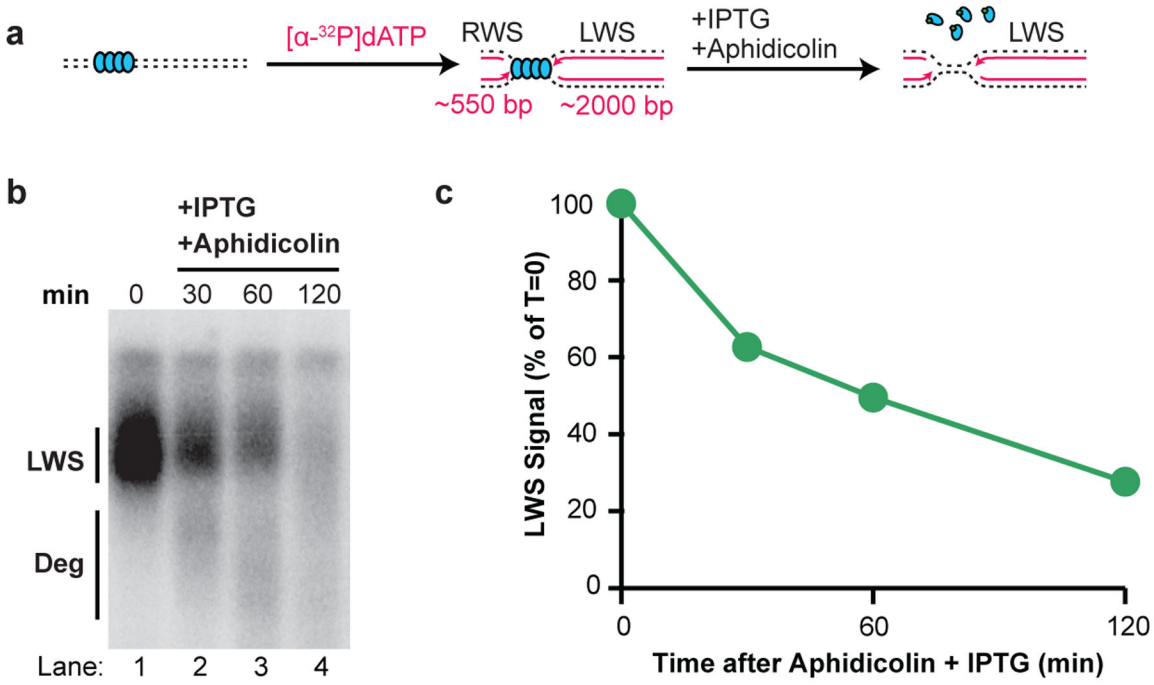

Fig 9. Denaturing gel analysis of nascent DNA strands

a, The DNA intermediates of DNA replication from **6a** were purified and digested with *AlwNI* to yield leftward strands (LWS) and rightward strands (RWS) of different sizes. **b**, Samples from **a** were separated on an alkaline denaturing gel and visualized by autoradiography. LWS strands are visible while the much smaller RWS are not. LWS decrease in abundance over time due to degradation and smears of degradation products ('deg') become visible. LWS are rapidly lost upon the onset of degradation because they decrease in size, which allows LWS abundance to serve as a read-out for the onset of degradation. **c**, Quantification of LWS from **b**. LWS decrease in abundance due to degradation. Note that degradation is more rapid than when total signal is monitored (compare to Fig 7C) because loss of LWS is a measure of the onset of degradation.

TABLE 1:

ABBREVIATIONS

Abbreviation	Definition
AMP	Ampicillin
ARS	ATP regenerating system
ATP	Adenosine 5’-triphosphate
AU	Arbitrary units
BSA	Bovine serum albumin
CAM	Chloramphenicol
CPK	Creatine phosphokinase
CTRL	Control
DMSO	Dimethyl sulfoxide
DPCs	DNA-protein cross-links
DTT	Dithiothreitol
DYs	Double Ys
EDTA	Ethylenediaminetetraacetic acid
ELB	Egg lysis buffer
HCl	Hydrochloric acid
HSS	High Speed Supernatant
ICLs	Interstrand cross-links
IPTG	Isopropyl β-D-1-thiogalactopyranoside
Kb	Kilobase
KCl	Potassium chloride
kDa	Kilodalton
KOH	Potassium hydroxide
lacO	Lac operator
LacR	Lac repressor
LB	Lysogeny broth
Lin	Linear
LWS	Leftward strands
MWCO	Molecular weight cut off
NaCl	Sodium chloride
NPE	Nucleoplasmic Extract
PAGE	Polyacrylamide gel electrophoresis
PC	Phosphocreatine
PPE	Personal protective equipment
RCF	Relative centrifugal force
RFs	Reversed forks
RIs	Replication intermediates
RNAse	Ribonuclease A

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Abbreviation	Definition
RWS	Rightward strands
rpm	Revolutions per minute
SDS	Sodium dodecyl sulfate
SOC	Super optimal medium with catabolic repressor
TBE	Tris-borate-ethylenediaminetetraacetic acid buffer
TCA	Trichloroacetic acid
Ys	Single replication forks

TABLE 2:

PLASMID DNA TEMPLATES

Plasmid Name	Size (kb)	Insert	Resistance Marker	Application
pBirAcm	7.901	BirA	CAM	Expresses BirA biotin ligase in *E. coli*. Biotinylates avi-tagged proteins.
pET11a [lacI::avi]	6.781	lacI::avi	AMP	Expresses Avi-tagged LacR in *E. coli*. Results in biotinylated LacR when co-expressed with BirA biotin ligase.
pJD91	3.652	*lacO*x24 (740 bp)	AMP	Can be used to assemble a 24x LacR array for *in vitro* DNA replication using a reversible replication barrier.
pJD100	4.402	*lacO*x48 (1500 bp)	AMP	Can be used to assemble a 48x LacR array for *in vitro* DNA replication using a reversible replication barrier.
pJD145	2.648	-	AMP	
pJD152	3.148	*lacO*x16 (548 bp)	AMP	Can be used to assemble a 16x LacR array for *in vitro* DNA replication using a reversible replication barrier.
pJD156	3.648	*lacO*x32 (1048 bp)	AMP	Can be used to assemble a 32x LacR array for *in vitro* DNA replication using a reversible replication barrier.
pJD161	4.527	*lacO*x25 (740 bp) - *Xho*I - *lacO*x25 (740 bp)	AMP	Can be used to assemble a 50x LacR array for *in vitro* DNA replication using a reversible replication barrier. Contains an *Xho*I site in the middle of the *lacO* array so that single replication forks can be analyzed by 2-D gel electrophoresis.
pJD194	4.561	Nb.*Bsm*I(lead) - CTCGTAAGGATACCGGT - Nb.*Bsm*I(lead) - *lacO*x25 (740 bp) - *Xho*I - *lacO*x25 (740 bp)	AMP	Allows for replication of leading strand DNA damage. Identical to pJD161 except for the addition of Nb.*Bsm*I sites flanking the sequence CTCGTAAGGATACCGGT. Treatment with Nb.*Bsm*I allows the sequence 5' – CATTCACCGGTATCCTTACGAGCG – 3' to be replaced with a modified oligonucleotide. When a LacR array is assembled the modified sequence is replicated on the leading strand template.
pJD195	4.561	Nb.*Bsm*I(lag) - ACCGGTATCCTTACGAG - Nb.*Bsm*I(lag) - *lacO*x25 (740 bp) - *Xho*I - *lacO*x25 (740 bp)	AMP	Allows for replication of lagging strand DNA damage. Identical to pJD194 except that the Nb.*Bsm*I sites and the intervening sequence are on the opposite strand. When a LacR array is assembled the modified sequence is replicated on the lagging strand template.

Table 3:

Troubleshooting table.

Procedure 1			
Step	**Problem**	**Possible Reason**	**Possible Solution**
97	Plasmid contains incorrect *lacO* array size	Plasmid was grown for too long	Grow plasmid for 16 h
		Plasmid was not grown with enough IPTG	Increase IPTG concentration in LB agar plates to 2-fold to 10 mM
		Plasmid was not stable at 37°C	Grow plasmid at 30°C
		Cloning strain expressed LacIQ	Express plasmid in NEB5α
126	Nicking yield is low	Nicked DNA is stuck in the DNA cleanup matrix	Follow the DNA Clean up kit procedure carefully
		Nicked DNA is stuck in the DNA cleanup matrix	Treat the nicking reaction with the appropriate concentration of Proteinase K
130		Insufficient nicking of plasmid DNA	Allow nicking reaction to continue for 3 h
	Ligation reaction has low efficiency is or is incomplete	Low quality of plasmids due to contamination during the plasmid preparation	Follow the miniprep procedures carefully and use new buffers in case of contamination
		Annealing reaction was unsuccessful	Check the expiration date of the ligase and all enzyme buffers
		Annealing reaction was unsuccessful	Do not exceed 72°C during annealing reaction
		Annealing reaction was unsuccessful	Use oligonucleotide at 100-fold molar excess to promote incorporation
132	*Age*I digest was unsuccessful or digested the plasmid	Incomplete digestion by the restriction enzyme	Check expiration date on enzyme and buffers
		Incomplete digestion by the restriction enzyme	Digest unmodified plasmid DNA with *Age*I-HF to verify it is functional
134	No DNA remains after the T5 Exonuclease digestion	The ligation was unsuccessful	Follow the procedures for the annealing and ligation reactions carefully
		Nuclease contamination	Follow all the steps carefully and acquire new reagents to avoid further contamination
Procedure 2			
9	Uneven signal between reactions	Improper mixing of HSS and DNA in licensing mix	Mix the licensing mix by using a P10 tip, avoid bubbles during mixing, and move the tip around the tube while pipetting to mix thoroughly
		Sampling different volumes	It is recommended to consistently sample at least 2 μl
9	Inconsistent results or visible differences in reaction volumes	Issues pipetting extracts, which are highly viscous	Use BrandTech pipettors and BrandTech P20 tips to pipet extracts as accurately as possible
11	Improper stalling at the LacR array	Too little LacR	Concentrate or re-purify LacR
		Incubation of LacR and DNA was too brief	Incubate LacR and DNA together for at least 1.5 h at room temperature
11	Forks not stalled at the LacR array prior to release	Low quality HSS and/or NPE	Quality control HSS and NPE as per[57]
12	Incomplete LacR release of replication forks	IPTG is not fully solubilized	Thaw IPTG (1 M in ELB (1X)) in room temperature water for at least 30 min before preparing any dilutions
		Low activity of IPTG	Check that Invitrogen IPTG is used to release LacR barriers
		Low activity of IPTG	Remake IPTG (1M in (ELB 1X))

Procedure 1			
Step	**Problem**	**Possible Reason**	**Possible Solution**
		Improper mixing of reaction with IPTG.	Use BrandTech pipettors and BrandTech P20 tips to mix reaction with IPTG as accurately as possible
12	Incomplete uncoupling of replication forks	Low effective Aphidicolin concentration	Add DMSO to 4% final reaction volume
25	Poor resolution of replication intermediates in native agarose gel	Insufficient SDS	Make extraction stop fresh before each use
Procedure 3			
23	Uneven recovery of DNA replication intermediates	Glycogen was not properly mixed	Vigorously pipet up and down glycogen and sodium acetate master mix down 5–10 times. Then vortex 30 sec - 60 sec
24, 56	Undigested species remain following treatment of purified replication intermediates with restriction enzyme	SDS or phenol contamination	Warm phenol/chloroform/isoamyl alcohol (25:24:1) to room temperature before use to prevent SDS precipitation carry over in subsequent steps.Check that the appropriate tubes are used for DNA extraction. If using other tubes than the recommended tubes, less aqueous phase my need to be recovered at each step to avoid phenol contamination
76	Denaturing gel resolution is low	Denaturing gel overheated during electrophoresis	Perform electrophoresis of denaturing gels at 4°C
Procedure 4			
4	Blurry gel images	Gel was not completely flat in the cassette	Expose gels with cassettes placed horizontally (flat) so gels do not shift
		Gel was not completely flat in the cassette	Place heavy books on top of cassettes while exposing
		Gel was not completely flat in the cassette	Add Whatman paper between the phosphoscreen and cassette
		Membrane or screen was in the incorrect orientation the cassette	Ensure the membrane is facing upwards contacting the phosphor (white) side of the phosphorimaging screen
4	Weak signal from gel image	[α-^{32}P]dATP is too old	Use fresh [α-^{32}P]dATP
		Phosphoscreen was not completely blanked	Ensure phosphoscreen is blanked for at least 10 min before use