Chapter 3

Preparation of Long Templates for RNA In Vitro Transcription by Recursive PCR

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Abstract

Preparing conventional DNA templates for in vitro RNA transcription involves PCR amplification of the DNA gene coding for the RNA of interest from plasmid or genomic DNA, subsequent amplification with primers containing a 5′ T7 promoter region, and confirmation of the amplified DNA sequence. Complications arise in applications where long, nonnative sequences are desired in the final RNA transcript. Here we describe a ligase-independent method for the preparation of long synthetic DNA templates for in vitro RNA transcription. In Recursive PCR, partially complementary DNA oligonucleotides coding for the RNA sequence of interest are annealed, extended into the full-length double-stranded DNA, and amplified in a single PCR. Long insertions, mutations, or deletions are accommodated prior to in vitro transcription by simple substitution of oligonucleotides.

Key words: In vitro transcription, RNA, DNA, Oligonucleotides, Synthetic DNA, Recursive PCR, Cloning, T7 promoter

1. Introduction

In a traditional polymerase chain reaction (PCR), an excess of oligonucleotide primers complementary to the 3′ ends of the double-stranded DNA fragment of interest (the DNA template), is combined with a small amount of DNA containing the template (1, 2). The source DNA is typically extracted from cells in the form of genomic or plasmid DNA. In the presence of deoxynucleoside triphosphates (dNTPs), Mg^{2+}, heat-stable DNA polymerase, and appropriate buffer, the template is amplified many orders of magnitude by repetitive cycling through temperatures optimal for denaturing, annealing of primers, and extension. Extension of the primers by DNA polymerase occurs in the 5′–3′ direction along the full length of the template (see Fig. 1a).

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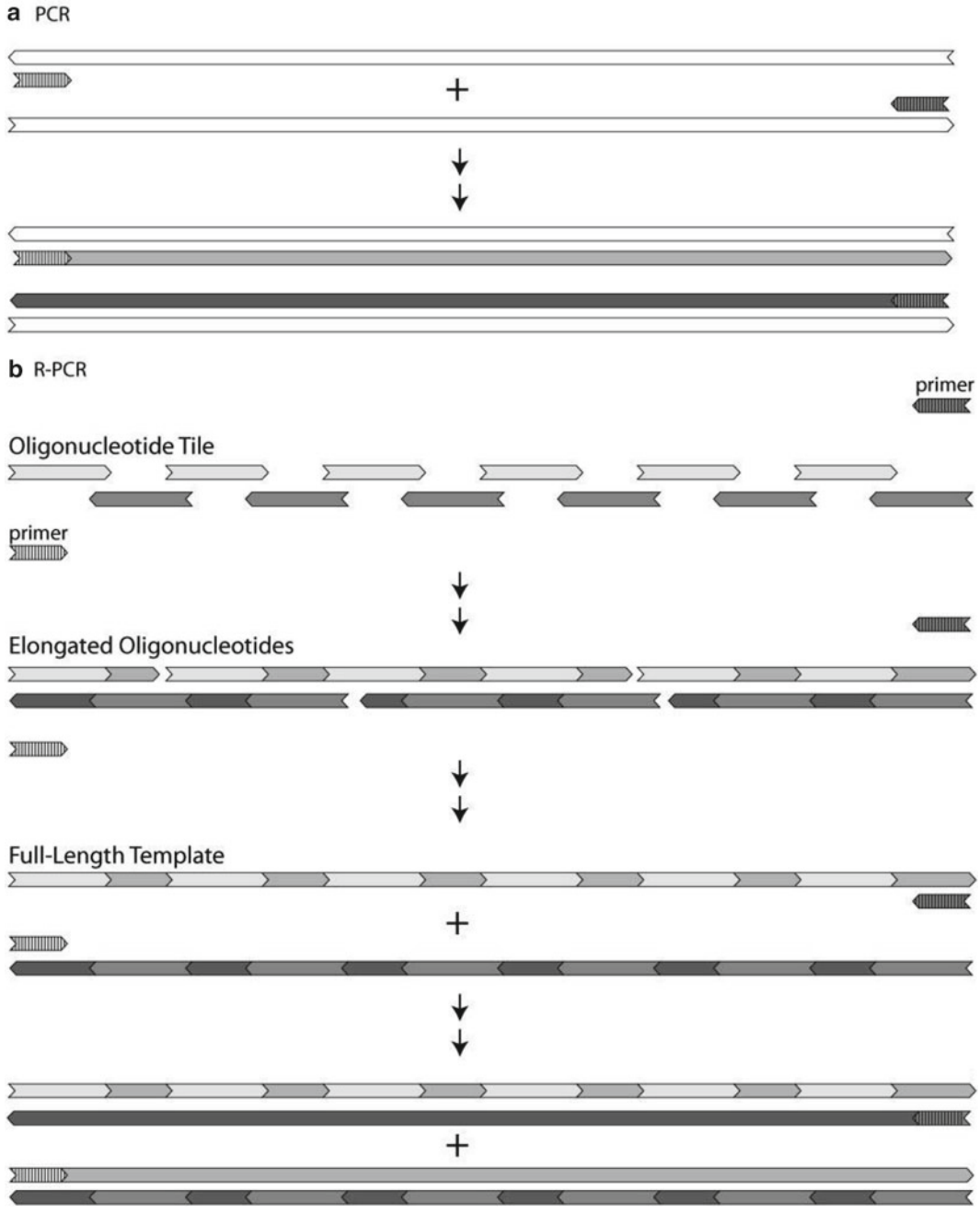

Fig. 1. Comparison of conventional and Recursive PCR. (**a**) A schematic diagram of a conventional PCR step illustrating the elongation of primers (*hashed arrows*) annealed to templates (*white arrows*). The direction of the chevron indicates the 5′–3′ directions. (**b**) A schematic diagram of an R-PCR. Initially an oligonucleotide tile is formed by synthetic oligonucleotides. The oligonucleotides are elongated and become successively longer with cycle number until some become full-length template. The added primers (*hashed arrows*) amplify the full-length template.

PCR can be used to construct long, double-stranded DNA from appropriate collections of long DNA oligonucleotides (oligos) in a process known as Recursive PCR (R-PCR). This method is named for its similarity to recursion in mathematics and computer science applications. R-PCR is also referred to as assembly PCR (3), parallel overlap assembly (4), or polymerase cycling assembly (5). R-PCR has the following advantages: (i) one-pot synthesis, (ii) a single dominant product, (iii) speed—often requires less than a day, (iv) material efficiency—chemical synthesis required for only half of the duplex DNA product (12), (v) modularity—facilitates nesting, modular synthesis, retrosynthesis, and synthesis of targeted libraries, and (vi) robustness—with no requirement for ligation.

PCR-based methods for splicing double-stranded DNA by overlap extension (6) anticipated R-PCR. The first published applications of R-PCR were the synthesis of a 522 base pair (bp) double-stranded DNA gene from ten oligos (7), and a 220 bp gene (8). Stemmer has used R-PCR for the single-step synthesis of DNA sequences up to 3 kb (3) and the approach has now achieved wide application (4, 9, 10). Genes can be obtained commercially; however R-PCR is a superior alternative to commercial genes under many circumstances. The benefits of R-PCR are greatest when fast turnaround is required, and a series of similar gene sequences is desired, whose extent of mutation, insertion, or deletion exceeds the range of a site-directed mutagenesis kit. Without R-PCR, such modification of commercially obtained genes can require de novo gene syntheses, many rounds of site-specific mutagenesis, or introduction of restriction endonuclease recognition sequences by mutagenesis followed by ligations. Modifying an R-PCR product can be as simple as swapping one or more oligos for one or more replacements, and repeating the R-PCR. Although synthesis of genes with substantial sequence modification by R-PCR adds complexity to the R-PCR oligo design process, these modifications can be quickly, reliably, and inexpensively incorporated.

In R-PCR, synthetic DNA oligos, with sequences identical to the sense and antisense strands of the target gene, are designed to form a tile. The tile contains alternating double-stranded and single-stranded regions. The 50–70mer oligos used to construct the tile are complementary on their ends giving duplex regions interleaved by single-stranded regions (see Fig. 1b). During an R-PCR, each 3′ terminus is extended, initially giving a mixture of products that coalesce to a single product as the number of PCR cycles increases (see Fig. 1b).

Here we focus on R-PCR/in vitro transcription (R-PCR/ivT)—the construction of DNA templates used for production of long RNAs by in vitro transcription. Preparation of a template for RNA in vitro transcription by R-PCR requires careful design of

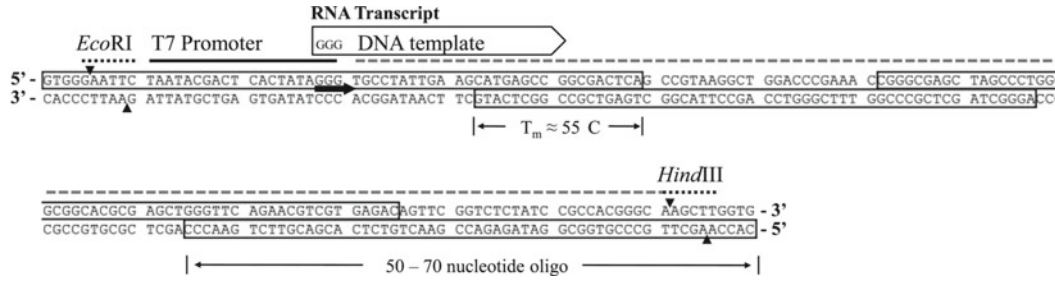

Fig. 2. Design of complementary DNA oligos for template synthesis by R-PCR. Stability nucleotides (5′-GTGG-3′), a unique restriction endonuclease recognition sequence (*Eco*RI), and the T7 promoter (*solid line*) are appended to the 5′ end of the gene sense strand. A second restriction site (*Hind*III) and stability nucleotides (5′-GGTG-3′) are appended to the 3′ end of the gene sense strand. The resulting sequence is divided into an even number of oligos, 50–70 nucleotides in length, alternating between the sense and antisense strands. Each oligo overlaps adjacent oligos by at least 15 bp, targeting an overlap melting temperature of 55°C. Transcription following digestion with the 3′ restriction endonuclease produces an RNA transcript that initiates at the tail of the arrow (shown between strands) and ends at the site of 3′ endonuclease cleavage.

the synthetic DNA oligos. First, the sequence of the target RNA fragment is converted to the sense DNA sequence using DNA analysis software such as Invitrogen's Vector NTI. A T7 polymerase recognition sequence (T7 promoter) is appended at the 5′ end of the sense sequence. To facilitate ligation to a vector, recognition sequences for unique restriction endonucleases, along with leading nucleotides necessary for efficient cleavage, are appended to the 5′ end of the T7 promoter and the 3′ end of the target sequence. The resulting DNA sequence is converted to a tile, by dividing it into partially overlapping oligos 50–70 nucleotides in length (see Fig. 2). The selected tile puts important constraints on future variants, and should therefore be chosen carefully. The nucleotides adjacent to anticipated insertions, mutations, or deletions are contained within the single-stranded regions (see Fig. 3). The oligos composing the tile, and additional 17–23mer primers for amplification of the full-length template, are obtained from a commercial vendor. The R-PCR is performed using a DNA polymerase with 3′–5′ proofreading activity, equal molar concentrations of the internal oligos, and a 10-fold molar excess of amplification primers.

Subsequent preparation of the DNA template is synonymous with preparation of a native template for in vitro transcription. The amplification product is purified from secondary products and primers by preparative gel electrophoresis, ligated to a vector containing the same endonuclease recognition sequences chosen for the template, and replicated in *Escherichia coli* (*E. coli*). The purified plasmid is confirmed by sequencing analysis to contain the correct DNA template and is linearized prior to in vitro transcription.

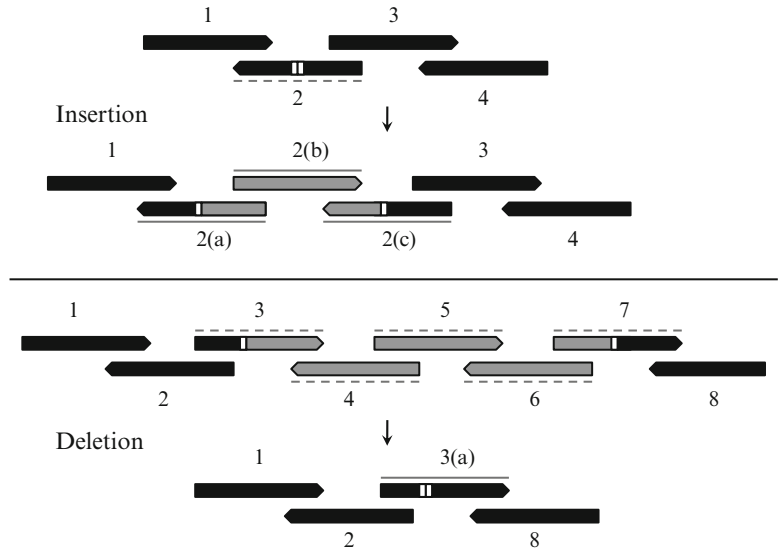

Fig. 3. Incorporating long insertions, mutations, and deletions by simple oligo substitution. Nucleotides flanking the planned modification (shown *white* in the schematic) are positioned within single-stranded regions. *Insertion*: Oligo 2 (*dashed line*) of a four-oligo template contains nucleotides flanking the planned insertion. Oligos 2(a), 2(b), and 2(c) (*solid line*) replace oligo 2 in the R-PCR to achieve the insertion (shown in *gray*). *Deletion*: Oligos 3, 4, 5, 6, 7 (*dashed line*) of an 8-oligo template contain a planned deletion (shown in *gray*). Oligo 3(a) (*solid line*) replaces oligos 3–7 in the R-PCR to achieve the deletion.

2. Materials

Prepare solutions using ultrapure water (18 MΩ cm at 25°C). Prepare and store all reagents at room temperature unless otherwise indicated.

2.1. R-PCR Components

1. DNA analysis/gene design software (see Note 1).
2. Thermocycler capable of performing a temperature gradient.
3. Nuclease-free water (see Note 2).
4. Autoclaved 1.5 mL microcentrifuge tubes (certified nuclease-free).
5. Autoclaved pipette tips (certified nuclease-free).
6. Autoclaved 0.2 mL PCR tubes (certified nuclease-free).
7. *Pfu* Polymerase (Stratagene), or an alternative DNA polymerase (see Note 3). Polymerases are usually supplied with 10× buffer. For *Pfu*: 200 mM Tris–HCl (pH 8.8 @ 25°C), 100 mM $(NH_4)_2SO_4$, 100 mM KCl, 20 mM $MgSO_4$, and 1% Triton X-100. Store at –20°C.

8. dNTP Mix: 25 mM dATP, 25 mM dCTP, 25 mM dGTP, and 25 mM dTTP. Mix 25 µL of each 100 mM stock of the four different dNTPs. Store at –20°C.
9. 2.5 µM 50–70mer oligos, commercially synthesized (see Note 4). Store at –20°C after rehydration.
10. 20 µM 17–27mer primers, commercially synthesized (see Note 4). Store at –20°C after rehydration.

2.2. Agarose Gel Electrophoresis Components

1. Horizontal agarose gel electrophoresis unit for 7×8 cm gels, and 1 and 1.5 mm thick combs with 6 and 8 wells.
2. Tris–acetate–ethylenediamine tetraacetic acid (EDTA) (TAE) Buffer (1×): 40 mM Tris–acetate and 1 mM EDTA. Use Molecular Biology Grade reagents. Can be conveniently purchased or prepared as a 40× stock: Dilute 50 mL of 40× TAE Buffer with 1,950 mL ultrapure water to prepare working concentration.
3. Agarose, LE, Analytical Grade.
4. Low-melting-temperature agarose for preparative DNA electrophoresis, such as SeaPlaque® Agarose.
5. GelStar® Nucleic Acid Gel Stain (Cambrex Bio Science Rockland, Inc.). Aliquot and store at –20°C.
6. DNA ladders for agarose gel electrophoresis: 100 bp and 1 kb DNA. Store at –20°C.
7. 1 M Tris (crystallized free base, Tris(hydroxymethyl)aminomethane): Dissolve 60.57 g in 500 mL ultrapure water. Adjust pH to 7.5 using HCl.
8. 0.5 M EDTA: Add 18.6 g to 100 mL ultrapure water and adjust pH to 8.0 using NaOH. Stir with heat until dissolved.
9. TE Buffer: 10 mM Tris, pH 7.5, 1 mM EDTA. Prepare by adding 5 mL of 1 M Tris and 1 mL of 0.5 M EDTA to 494 mL ultrapure water.
10. 6× Loading Dye, Blue: 2.5% Ficoll 400, 11 mM EDTA, 3.3 mM Tris–HCl, 0.017% SDS, and 0.015% Bromophenol Blue, pH 8.0 at 25°C. Store at –20°C.
11. Loading Dye: Add 100 µL of 6× Loading Dye, Blue, to 200 µL TE buffer. Store at –20°C.

2.3. DNA Purification Components

1. Agarose gel DNA extraction kit (see Note 5).
2. PCR and enzymatic reaction DNA purification kit (see Note 5).

2.4. Cloning Components

1. *Eco*RI and *Hind*III Restriction Endonucleases (20,000 units/mL), supplied with corresponding 10× reaction buffers, or appropriate alternatives (see Note 6).
2. pUC19 vector, or an appropriate alternative (see Note 6).

3. Phosphatase (5,000 units/mL) capable of heat inactivation at 65°C, supplied with 10× Phosphatase Buffer.
4. Quick Ligation Kit.
5. Chemically competent *E. coli* cells, such as DH5α (see Note 7).
6. Sterile inoculating loops or autoclaved toothpicks.
7. Lysogeny Broth-Ampicillin (LB-Amp) agar plates (with 50 μg ampicillin per mL agar).
8. LB medium.
9. 100 mg/mL Ampicillin.
10. Culture tubes.
11. "Mini" scale plasmid DNA preparation kit capable of purifying up to 20 μg of high-copy plasmid from 2 mL overnight *E. coli* cultures in LB, or alternative plasmid purification method.
12. "Maxi" scale plasmid DNA preparation kit capable of purifying up to 500 μg of high-copy plasmid from 100 mL overnight *E. coli* cultures in LB, or alternative plasmid purification method (for example, see Chapter 4).

3. Methods

3.1. Synthetic Oligonucleotide Design

1. Convert the sequence of the desired RNA transcript to its corresponding sense DNA sequence (see Note 8). Import the DNA sequence to the sequence editor of a DNA analysis/gene design software program (see Note 1).
2. Search the sequence for recognition sequences of common, commercially available restriction endonucleases. Take note of any that appear within the template, and eliminate these from consideration when choosing a vector and cloning site.
3. Choose a ligation vector and cloning site within the vector (see Note 9). A commonly used vector is pUC19 (see Note 10). Two commonly used restriction sites are *Eco*RI (5′-GAATTC-3′) and *Hind*III (5′-AAGCTT-3′) (see Note 6).
4. Using the software sequence editor, append the sense sequence of the first restriction site followed by a T7 promoter sequence (5′-TAA TAC GAC TCA CTA TAG GG-3′) to the 5′ end of the DNA sense template (see Note 11). Append the second restriction site to the 3′ end of the DNA sense sequence.
5. Add four stability bases (e.g., GCTG or GGTG) to the 5′ and 3′ ends of the resulting sequence. These additional nucleotides foster stable duplex formation and are necessary at the terminal regions for efficient cleavage by many restriction endonucleases.

6. Beginning with the sense strand, divide the target sequence into an *even* number of oligos, each 50–70 nucleotides in length (see Note 12). The oligos must alternate between the sense and antisense strands. Adjacent sense and antisense oligos should overlap by at least 15 complementary nucleotides (Fig. 2). Since the first oligo is defined on the sense strand, and an even number of oligos is necessary, the last oligo is defined on the antisense strand (see Note 13). Nucleotides adjacent to any planned modifications (insertions, mutations, or deletions) by oligo substitution in the R-PCR must appear within the nonoverlapping regions (see Note 14).
7. Using the thermodynamic property analysis tool that accompanies most DNA analysis/gene design software programs, adjust the lengths of the sense and antisense oligos to achieve an estimated *overlap melting temperature* of 52–58°C (see Note 15).
8. Using DNA analysis software, check individual oligo overlap sequences for potential interfering secondary structure by searching the full target sequence for each specific overlap sequence, using a mismatch tolerance appropriate for the length of the overlap. If problematic secondary structure is found, lengthen oligos to increase specificity or shift the overlap away from secondary structure if possible. Ideally, overlapping regions are 17–20 bp in length or the length required to produce a melting temperature of 52–58°C.
9. Design standard forward and reverse amplification primers (17–27mer) complementary to the 3′ ends of the full-length DNA template (see Note 16). Here the full-length template includes the outermost stability nucleotides, the restriction sequences, and the T7 promoter. Both oligos and amplification primers can be procured lyophilized in tubes.

3.2. The R-PCR

Perform all procedures at room temperature unless otherwise specified.

1. Commercial oligos and primers are obtained in lyophilized form in tubes accompanied by a datasheet specifying the actual mass and number of moles delivered. Follow the supplier's directions for hydrating the lyophilized product to a concentration of 100 μM (strand) in nuclease-free water. After resuspension, check the final concentration with a UV spectrophotometer. Prepare 2.5 μM working stocks of each oligo, and 20 μM working stocks of each primer in nuclease-free water. Freeze the 100 μM stocks at −20°C and avoid frequent freeze/thaw cycling (see Note 17).
2. For the initial R-PCR, multiple reactions are generally prepared to facilitate PCR across a gradient of annealing temperatures, spanning a range of 45–65°C (see Note 18 and Fig. 4).

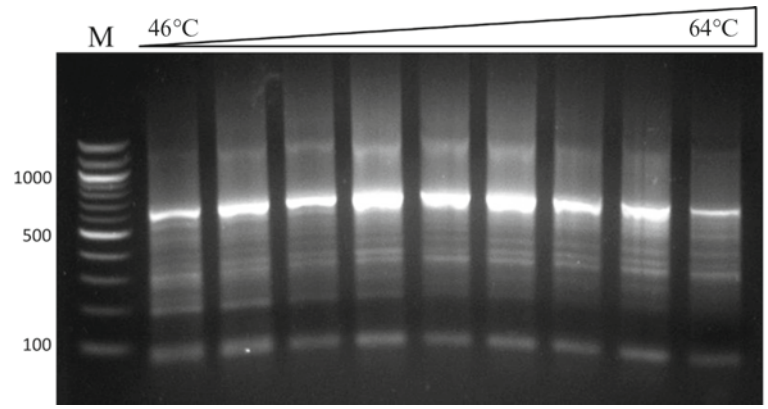

Fig. 4. Analytical gel (2% agarose) of replicate R-PCRs, showing successful synthesis of a 670 bp product amplified from 16 oligos over a range of annealing temperatures (46–64°C). Product amplification is optimal among *lane 5* (53°C), *lane 6* (55°C), and *lane 7* (58°C), centered around the design overlap melting temperature (~55°). Multiple secondary amplification products are almost always observed. Excess oligos appear near the 100 bp (smallest) marker.

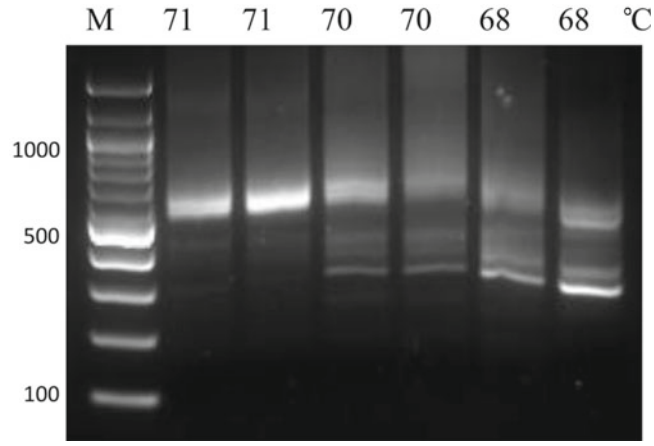

Fig. 5. Analytical gel (2% agarose) of replicate dsDNA "stitching" PCRs. Two double-stranded DNA fragments (190 and 480 bp, previously synthesized by R-PCR), containing a single 17 nucleotide overlapping region, amplified over a range of annealing temperatures: *lanes 2–3* (71°C), *lanes 4–5* (70°C), *lanes 6–7* (68°C). *Lanes 2* and *3* illustrate successful amplification of the ~650 bp target.

Optimal annealing usually occurs around 55°C; however, this gradient can prove essential for optimizing amplification of templates with difficult secondary structure regions (Fig. 5). Prepare an equimolar master mix of the DNA oligos from the working stocks by combining 5.5 μL of each 2.5 μM oligo in a 1.5 mL sterile tube for every five PCRs. Assemble 50 μL reaction volumes in the order shown in Table 1 (see Notes 19 and 20).

Table 1
Components and assembly of the R-PCR

Volume	Component	Final concentration
To 50 µL total	Nuclease-free water	
5 µL	10× Polymerase reaction buffer	1×
1.25 µL	20 µM 5′ amplification primer	500 nM[a]
1.25 µL	20 µM 3′ amplification primer	500 nM[a]
0.5 µL	25 mM dNTPs (each dNTP)	250 µM
1 µL per oligo	DNA oligo master mix	50 nM each oligo[a]
1 µL	*Pfu* DNA polymerase or alternative (see Note 3)	~3 units

[a] See Note 20

Table 2
R-PCR cycling parameters

Step	Cycles	Temperature (°C)	Time (min)	Description
1	1	95	2	Initial denaturation
2	30	95 59.5 ± 9 gradient 72	1 1 1 min per 1,000 nucleotides	Cyclic denaturation Annealing Extension
3	1	72	2	Final extension
4	1	4	HOLD	

3. Program the thermocycler to cycle through the conditions shown in Table 2 (see Note 21). For the gradient annealing step, target annealing at temperatures of 53, 55, 59, 62, and 65°C (see Note 18). After thermocycling, the PCR mixture can be held at 4°C overnight or stored at –20°C.
4. Prepare an analytical agarose gel of appropriate percentage for the length of the anticipated amplification product (generally, this will be a 1.5–2% for the product size range appropriate for the R-PCR). For a 2% gel, add 1 g Agarose LE to 50 mL of 1× TAE buffer (see Note 22) in a 250 mL Erlenmeyer flask. Dissolve for 2–5 min in a microwave or heat with stirring on a hot plate. Boil until fully dissolved (less than 5 min). Prepare a horizontal gel electrophoresis apparatus by inserting the gel-casting tray sideways to seal the tray rubber against the interior

walls of the apparatus. Remove the agarose solution from heat. Before pouring, add 0.5 μL GELSTAR® Nucleic Acid Stain to the bottom of the gel-casting tray (see Note 23). Pour agarose into the casting tray as soon as it ceases to boil, to a thickness of approximately 0.5 cm. Using a pipette tip, mix the GELSTAR® into the agarose by vigorously tracing a grid of vertical and horizontal lines through the agarose. Mix well, but avoid unnecessary introduction of bubbles (see Note 24). Insert the appropriate 1 mm thick well-forming comb and allow the gel to solidify for 15–20 min.

5. Prepare a 100 bp DNA ladder and samples of each R-PCR for electrophoresis by adding 1 μL of each to 4 μL of the Loading Dye, Blue (see Note 25).
6. Check that the agarose has solidified (it should be cloudy in appearance and firm). Carefully remove the gel-casting tray containing the gel and place it in the gel apparatus in a position such that the comb is positioned nearest the black (negative) terminal of the apparatus. Submerge the gel in ~400 mL of 1× TAE buffer. Load each of the 5 μL samples prepared in the previous step to an individual well. Plug the electrodes into the power supply and run at 140 V for 20–30 min or until the dye front traverses ~70% the length of the gel.
7. Stop the power supply and remove the gel with cast from the electrophoresis apparatus.
8. Image the gel with a 302 or 312 nm UV transilluminator for dye excitation.
9. Compare amplification products with the molecular weight ladder. A bright and dominant band, approximately the same size as the calculated total length of the desired DNA template, indicates a successful R-PCR (Fig. 4). Multiple secondary amplification products (less dominant bands of varying size) are expected in R-PCR.

3.3. Purification of the DNA Template

1. Purify the desired amplification product from secondary products by preparative gel electrophoresis of the PCR product mixture. Cast a preparative (low melting) agarose gel of appropriate percentage for the length of the anticipated amplification product (1.5–2%, as for the diagnostic agarose gel). For a 2% gel, dissolve 2 g of SeaPlaque® GTG agarose in 100 mL of 1× TAE buffer in a 250 mL Erlenmeyer flask. Careful—the melting temperature of SeaPlaque® agarose is much lower than that of Agarose LE. Watch carefully while boiling to prevent boil over. Prepare a gel-casting tray as before and just before pouring, place 0.75 μL GELSTAR® in the bottom of the casting tray (see Note 23). Pour approximately 75 mL of agarose into the tray or to a thickness of 1 cm and mix in GELSTAR® as previously described. Insert the 1.5 mm side of a 6-well comb

into the agarose (see Note 26). Allow 45–60 min for the preparative gel to solidify.

2. Prepare a 100 bp molecular weight ladder by adding 1.8 μL of ladder to 18.2 μL loading dye.
3. Add 4 μL of 6× loading dye directly to each PCR, and mix well.
4. When the gel has fully solidified, submerge it in 1× TAE buffer (see Note 22). Load the molecular weight ladder–dye mix, and the full volume of each PCR–dye mix to each of the six wells. Run at 109 V for 30–45 min or until the dye front traverses ~70% the length of the gel.
5. Preheat a heating block to 55°C. Pipette 500 μL of nuclease-free water to a 1.5 mL microcentrifuge tube and place it in the heating block.
6. Image the gel with a UV transilluminator, taking care to minimize exposure time to the UV source.
7. Compare the bands on the preparative gel with the molecular weight ladder, and with the analytical gel. Secondary products that were not visible on the analytical gel may be visible on the preparative gel due to the large mass of DNA loaded. Due to a dye quenching effect, highly concentrated DNA does not stain well, and may appear as slight "parentheses" at the edges of the lane, with no definitive band visible (Fig. 6). Preparative gels

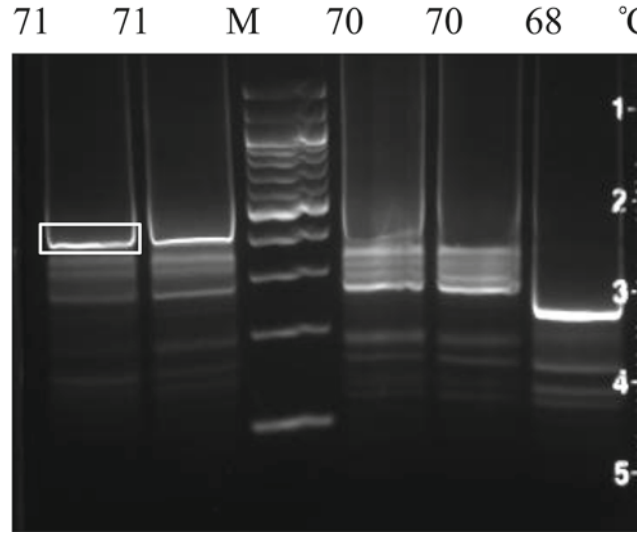

Fig. 6. Preparative gel (2% agarose) of the "stitching" PCRs shown in Fig. 5, with the second 68°C PCR omitted. The entire 50 mL volume of each PCR is loaded to a well. Due to a dye-quenching effect, the ~650 bp product band appears as "parentheses" along the sides of the well in *lanes 1* and *2*. In this preparative gel, the ~650 bp product (as seen on the analytical gel) runs between the 400 and 500 bp markers. The ~650 bp product to be excised from *lane 1* is enclosed by a box.

may also run differently relative to the molecular weight marker as compared to analytical gels. Carefully analyze and identify the location of the desired amplification product before proceeding (see Note 27).

8. Return the gel to the transilluminator and excise a minimal gel slice from each lane containing the amplification product using a razor blade. Place the gel slices in sterile 1.5 mL microcentrifuge tubes.
9. Add the volume of agarose dissolving/solubilizing buffer recommended by the gel extraction kit manufacturer to each gel slice and place the tubes in the heating block for 20 min (or until the gel slice is completely dissolved), mixing every 5 min (see Note 28).
10. Follow the gel DNA extraction kit protocol to purify the DNA from the dissolved agarose. Elute DNA in 55°C nuclease-free water. Avoid elution of DNA in a total volume greater than 30 μL.
11. Measure the UV absorbance of each eluted DNA template at 260 nm. Calculate the w/v concentration by multiplying the absorbance at 260 nm by the dilution factor (if any) and the 50 ng/μL conversion factor for dsDNA. Store at –20°C unless proceeding immediately (see Note 29).

3.4. Sequential Digest of the DNA Template and Host Vector

1. Calculate the volume of gel-purified DNA containing 200–500 ng of DNA template and transfer this volume to a sterile 1.5 mL microcentrifuge tube. Similarly, calculate the volume containing 500 ng of vector (see Note 10) and transfer that volume to a second microcentrifuge tube. Bring the total volume in each tube to 17.5 μL with nuclease-free water. Add 2 μL of 10× Restriction Enzyme Buffer and 0.5 μL of *Hind*III restriction endonuclease (or digest with an appropriate alternative). Incubate at 37°C for 1 h (see Note 30).
2. Purify the DNA from the enzyme and buffer using the PCR and enzymatic reaction DNA purification kit (see Note 31). Target an elution volume ≤17.5 μL.
3. Estimate the final elution volume using a pipette. To each tube, add nuclease-free water to a total volume of 17.5 μL. Add 2 μL of 10× *Eco*RI Buffer and 0.5 μL of *Eco*RI restriction endonuclease (or digest with an appropriate alternative). Incubate at 37°C for 1 h.
4. Preheat a heating block to 65°C. Remove tubes from the incubator. Add 24 μL nuclease-free water, 5 μL 10× Phosphatase Buffer, and 1 μL Phosphatase to the *vector sample only*. Incubate at 37°C for 1 h.
5. Remove tubes from the incubator and heat-inactivate at 65°C for 20 min.

6. Purify the digested template and vector from the phosphatase buffer using the PCR and enzymatic reaction purification kit. Elute DNA in 65°C nuclease-free water. Avoid elution of DNA in a total volume greater than 30 μL.
7. Measure the UV absorbance of each eluted DNA template at 260 nm, calculating w/v as described in Subheading 3.3. Store digested, purified template and vector at 4°C.

3.5. Ligation, Transformation, and Sequence Confirmation

1. Place two new LB-Amp plates (or LB plates with the appropriate selective antibiotic for the chosen vector) in a 37°C incubator. Add 50 ng of digested vector to each of the two sterile 1.5 mL microcentrifuge tubes. Estimate the mass required for a threefold molar excess of template insert by calculating the size of the insert as a fraction of the size of the vector and multiplying by 150. Add the calculated mass of insert to one of the two tubes containing 50 ng of vector, and bring the volume up to 10 μL (the ligation reaction). To the second of the two tubes containing 50 ng of vector, only add nuclease-free water to bring the volume up to 10 μL (background control). Add 10 μL of Quick Ligation Reaction Buffer and 1 μL of Quick T4 DNA Ligase enzyme mix to both tubes. Incubate at room temperature for 15 min (see Note 32).
2. Allow chemically competent cells to thaw on ice for 15–20 min. Transfer 5 μL of each ligation reaction to a sterile 1.5 mL microcentrifuge tube. Chill on ice for 5 min. Add 100 μL of competent cells to each 5 μL of ligation reaction. Incubate on ice for 45 min.
3. Remove the LB Amp plates from the incubator. Plate the entire 105 μL reaction (see Note 33) and incubate at 37°C for 16 h. Discard any remaining competent cells as biohazard waste.
4. Compare the number of colonies on background control plate to the ligation plate. The number of colonies on the ligation plate, relative to the number of colonies on the background control plate, dictates the recommended number of colonies to screen for plasmid containing the DNA template (see Note 34). Prepare a colony PCR master mix for screening in total reaction volumes of 10 μL. For every four colonies to be screened, combine all components for a single 50 μL PCR as listed in Table 1, omitting only the DNA oligos. Aliquot 10 μL to each of the four PCR tubes.
5. Prepare a new LB Amp plate for inoculation by sectioning into four parts. Using an inoculating loop or a sterile toothpick, touch a single colony on the ligation plate, touch the loop to one section of the prepared LB Amp plate (pre-warmed at 37°C), and then swirl the loop in one of the prepared PCR aliquots. Associate labels on the PCR tube with the plate freshly inoculated with the corresponding single colony. Incubate the

LB Amp plate(s) at 37°C for 16 h, and then store at 4°C, inverted and wrapped in parafilm.

6. Change the initial 95°C denaturing duration on the thermocycler to 5 min to facilitate cell lysis. Otherwise, cycle the PCR consistent with Table 2.
7. Prepare and load an analytical gel of the colony PCR as previously described for the R-PCR (see Subheading 3.2). Image the gel and evaluate for amplification products of similar size to the target DNA template.
8. From the LB Amp plate inoculated concurrent with preparation for the colony PCR, inoculate any colonies positive for DNA template (confirmed by analytical gel electrophoresis) into 2 mL of LB broth with 6.25 μL of 100 mg/mL ampicillin (or the appropriate selective antibiotic for the chosen vector). Culture for 16 h at 37°C with vigorous shaking.
9. Transfer 1.4 mL of each culture to a sterile 1.5 mL microcentrifuge tube. Harvest cells at 6,800 × *g* for 3 min.
10. Prepare mini-scale plasmid purification kit buffers and purify plasmid as per the manufacturer's protocol. Elute DNA with 50 μL of 65°C nuclease-free water.
11. Measure the UV absorbance of each eluted plasmid sample. Prepare 15 μL of plasmid at a concentration of 100–300 ng/μL and send for sequencing with plasmid-specific primers flanking the cloning site (for a template ligated to the multiple cloning site (MCS) of pUC19 vector, use M13F and M13R universal primers).
12. Compare plasmid sequencing results with the full-length DNA template designed in Subheading 3.1 using DNA analysis software.

3.6. Large-Scale Replication of the Plasmid in E. coli

1. Autoclave 100 mL LB Broth in each of the two 500 mL culture flasks for step 3.
2. From the selective media plate inoculated concurrent with preparation for the colony PCR, prepare two culture tubes with 2 mL of LB broth and 6.25 μL of 100 mg/mL ampicillin (or the appropriate selective antibiotic for the chosen vector). Inoculate each with a single colony containing plasmid with the confirmed DNA template sequence from Subheading 3.5. Culture for 8 h at 37°C with vigorous shaking.
3. After 8 h, add 312 μL of 100 mg/mL ampicillin (or the appropriate selective antibiotic for the chosen vector) to each 100 mL of autoclaved LB Broth. Inoculate each with 1 mL of the starter culture. Culture for 16 h at 37°C with vigorous shaking.
4. Harvest cells at 4°C for 15 min at 6,000 × *g*. Purify plasmids per Maxi-scale plasmid purification kit as per the manufacturer's

protocol (see Note 35). Resuspend pellet in 500 µL nuclease-free water, taking care to thoroughly wash down the sides of the tube. Transfer resuspended plasmid to a sterile 1.5 mL microcentrifuge tube.

5. Measure the UV absorbance of the resuspended plasmid. Dilute and aliquot freezer stocks, if desired, but do not dilute below 150 ng/µL.

3.7. Plasmid Digestion at the 3′ End of the DNA Template and Purification

1. Calculate the volume of resuspended plasmid from Subheading 3.6 containing 1 µg of plasmid DNA and transfer this volume to a 1.5 mL microcentrifuge tube. Add 2 µL of 10× Restriction Enzyme Buffer and 0.5 µL of *Hind*III restriction endonuclease (or digest with an appropriate 3′ alternative). Incubate at 37°C for 2 h.
2. Heat inactivate the restriction endonuclease at 65°C for 20 min.
3. Remove tube from the heating block and allow it to cool on the bench for several minutes. Purify the DNA from the enzyme and buffer using the PCR and enzymatic reaction DNA purification kit. Elute DNA in 65°C nuclease-free water.
4. The linearized, purified plasmid containing the DNA template is now ready for in vitro RNA transcription.

4. Notes

1. Design of complementary oligos is best performed in a software program capable of showing both the sense and antisense DNA strands, calculating the melting temperature of selected regions, and recognizing common restriction sites. One example is Invitrogen's Vector NTI, though many other programs are available.
2. Nuclease-free water can be obtained from most common molecular biology supply companies, or alternately made by treating ultrapure water with diethylpyrocarbonate (DEPC). Add 0.5 mL of DEPC to 1 L ultrapure water (to 0.05%), mix well, and incubate for 2 h at 37°C. Autoclave to hydrolyze the DEPC before use.
3. While *Pfu* is routinely used by our lab for R-PCR, Freeland and coworkers (11) did not observe any full-length product when using *Pfu*, and instead reported much higher performance from *KOD* XL and *KOD* Hifi DNA polymerases which also act significantly more quickly, suggesting the potential for routine synthesis of genes up to 1 kb in under an hour. Our initial R-PCRs with Phusion® High-Fidelity DNA Polymerase and buffer (as per the manufacturer's instruction) suggest

that amplification may be superior to *Pfu* for some R-PCR applications. Alternatively, Vent DNA polymerase is reported to be effective in R-PCR (7). Xiong used *pyrobest Taq* DNA polymerase to splice double-stranded DNA fragments of intermediate length (400–500 bp), generated by R-PCR using *Pfu*, into DNA templates of multiple kb lengths (12). If using an alternative DNA polymerase, be sure to use the appropriate reaction buffer and cycling conditions.

4. Oligos are commercially available from many different companies. We order DNA oligos and primers for R-PCR at the 50 nmol synthesis scale with high-purity salt-free purification.
5. We prefer Agarose Dissolving Buffer, DNA Wash Buffer, and Zymo-Spin I Columns purchased together in the Zymoclean™ Gel DNA Recovery Kit from Zymo Research. DNA Binding Buffer, DNA Wash Buffer, and Zymo-Spin I Columns can also be purchased together in the DNA Clean and Concentrator™-5 kit from Zymo Research. Alternatives include the QIAquick® PCR Purification Kit or QIAquick® Gel Extraction Kit from Qiagen.
6. Most commercially available plasmids contain an MCS, or a region dense in common restriction sites often unique within the plasmid. Cloning into the MCS is often advantageous due to its well-defined sequence and widespread commercial availability of flanking primers. Two different restriction sites should be selected for the cloning site to ensure that the insert is ligated into the vector in the desired orientation. These sites should occur only once in the entirety of the vector sequence, and not appear in the DNA template. For convenience, endonucleases capable of double digest and heat inactivation should be selected wherever possible. The length of the plasmid relative to the length of the DNA template after ligation should be minimized. Long plasmids, relative to the size of the template to be transcribed, can reduce the efficiency of in vitro transcription. Selection of a vector 1.5–5 times the length of the DNA template is generally a good choice. If a T7 promoter is designed into the DNA template, the vector chosen for ligation should not contain its own T7 promoter region. Freeland used *Hind*III and *Bam*HI to clone their gene of interest into pUC19, but do not specify which restriction site was placed at which end of the gene (11). We have found that the double digest finder on New England Biolabs' Web site is an excellent resource for identification of compatible restriction endonucleases. If using alternate restriction enzymes, use the appropriate digestion buffers for the selected enzymes.
7. Alternatively, competent *E. coli* cells can be prepared from a fresh overnight culture. Inoculate 50 mL LB media with ~1 mL overnight culture. Incubate at 37°C for about 1 h, or until an

OD_{550nm} of 0.45–0.6 is reached (no higher). Incubate on ice for 15 min, and then pellet cells for 15 min at 4°C. Resuspend pellet in 16 mL RF1 Buffer: 100 mM RbCl, 50 mM MnCl, 30 mM KOAc, 10 mM CaCl, and 15% glycerol (v/v); adjust pH to 5.8 with 0.2 M acetic acid, filter sterilize, and store at 4°C. Incubate on ice for 30 min. Pellet cells at 3,000 × *g* for 15 min at 4°C. Working on ice, resuspend pellet in 4 mL RF2 Buffer: 10 mM MOPS (pH 6), 10 mM RbCl, 75 mM CaCl, and 15% glycerol (v/v); filter sterilize, and store at 4°C. Aliquot 300 μL to a prechilled, sterile 0.65 mL tube. Freeze in liquid nitrogen or a dry-ice ethanol bath. Repeat for the remaining volume. Store at –80°C.

8. This is easily done using the "Find and Replace" function of any text editor (replace all "U"s with "T"s), or by easily searchable online transcription tools.
9. Ligation of the DNA template, though time consuming, reduces transcription of undesirable secondary amplification products produced by PCR, and facilitates large-scale production of the DNA template.
10. pUC19 plasmid vector is used in this example; however alternate vectors such as the phagemid pGEM3Zf(+) have been used as well (7).
11. The specified T7 promoter is one that we have used successfully. The transcription start site is the first G residue encountered after the TATA portion of the promoter (see Fig. 2), such that this and all nucleotides after it will be incorporated into the RNA sequence by T7 RNA polymerase. Depending on the application, it may be necessary to alter the target RNA sequence to account for the presence of these initial guanosines in the transcribed RNA. There are multiple iterations of the T7 promoter published. One variation is the φ2.5 promoter, 5′-TAATACGACTCACTATTAGGG—3′, where the underlined adenosine is the initiating nucleotide. This promoter was shown to increase transcription efficiency and 5′ end homogeneity of the transcribed RNA (13).
12. Prodromou and Pearl recommend oligos 54–86 nucleotides long for optimal yields and minimal errors (7), while Freeland successfully utilized 40 nucleotide oligos with unoptimized 20 nucleotide overlaps, using an annealing temperature of 52°C (11).
13. When designing oligos for R-PCR, it is helpful to print a copy of the DNA sequence, showing both the sense and antisense sequence, for preliminary delineation of oligos. A simple method for delineation is to initially specify homogeneous overlaps of 15 bp and then to calculate the resulting number and length oligos required. First, calculate the number of

oligos needed for a sequence where Length$T$ is the number of base pairs in the full-length DNA template, including stability bases, restriction sites, and T7 promoter:

$$\text{Number of oligos} = (\text{Length}_T / 40) - 0.375.$$

Round the number of oligos to the nearest *even* integer and then calculate the oligo length:

$$\text{Length of each oligo} = [(\text{Length}_T - 15)/(\text{Number of Oligos})] + 15.$$

Design initiates with the 5′ side of the sense sequence. If the length of each oligo (calculated above) is 55 bp, delineate the first 55 nucleotides of the *sense strand* by outlining or highlighting. This is preliminary oligo 1 (sense). Immediately after the last nucleotide in oligo 1, move vertically to the antisense strand. Backtrack 15 nucleotides, along the *antisense*, and delineate the 15-nucleotide overlap with oligo 1 plus the following (contiguous) 40 nucleotides of the antisense as oligo 2 (antisense). Move vertically to the sense sequence and repeat. Continue alternating sense and antisense, until preliminary oligos with complementary overlaps have been delineated for the entire DNA template. Because we are working with an even number of oligos, the last oligo delineated should (and must) be on the antisense strand.

14. When designing oligos for modifications by oligo substitution, the contiguous region to be modified must be located entirely within the nonoverlapping region of an oligo, which is roughly 20 bp in length. If the desired modification is longer, consider substitution of multiple oligos. Note that substitutions of one oligo can be made by another single oligo, three oligos, five oligos, etc. An odd number is necessary to retain the pattern of alternating sense and antisense oligos. Alternatively, it may be appropriate to extend the length of the oligo to be substituted up to 65 nucleotides. Longer oligos have a greater tendency to form interfering secondary structure, complicating synthesis and purification by the manufacturer, as well as in the R-PCR.
15. Overlapping regions of oligos should be evaluated in the same manner as amplification primers. For example, avoid overlap sequences likely to pair with an undesired overlap, seek a 3′ GC clamp, and avoid dimers and difficult secondary structure regions. DNA analysis software can be used to search for regions of nonspecific priming and secondary structure.
16. R-PCR is most efficient for DNA templates of 150–700 bp in length, with little to no secondary structure. For templates with regions of significant secondary structure or of length greater than 700 bp, we find it prudent to design additional primers for amplification of the DNA template in parts, retaining a common overlapping region. If the initial R-PCR fails to

amplify the full DNA template in a single reaction, then the oligos can be split into two separate R-PCRs, amplified individually into double-stranded DNA, and subsequently spliced into the full-length gene by dsDNA overlap extension (6, 12).

17. Careful preparation of template primer working stocks is critical. Template primers must be added to the PCR in equal molar concentrations. Take care to ensure that lyophilized primers are fully hydrated and resuspended prior to preparation of working stocks. Wu recommends resuspension of oligos in 10 mM Tris–HCl (pH 8.5) (11). For synthesis of an individual gene, a single working stock volume of 100 μL for each primer and oligo is usually more than sufficient. For more elaborate applications aliquot the 100 μM stocks in volumes anticipatory of future working stock preparation to avoid freeze/thaw cycling.
18. If this annealing temperature gradient does not produce the desired product, it may be helpful to further increase the annealing temperatures up to 71°C, particularly when the target sequence contains difficult secondary structure regions, which often interfere with R-PCR. However, in other reported cases, annealing temperature has no significant effect on R-PCR product yield. Freeland observed no perceivable difference when varying the annealing temperature from 48 to 62°C using *KOD* XL and *KOD* HiFi DNA polymerases, and consistently achieved success with a 52°C annealing temperature (11).
19. When assembling PCRs with a polymerase that exhibits 3′–5′ exonuclease activity (such as *Pfu*), the order of addition of the enzyme relative to the dNTPs is critical. Add the polymerase last to prevent possible degradation of primers and template.
20. Prodromou (7) achieved synthesis of their desired product using 0.2–0.3 pmol of each oligo in combination with 20–30 pmol of each amplification primer in a 100 μL total volume reaction with Vent DNA polymerase. They reported that the 100-fold difference proved crucial in achieving the target product. However, Freeland reports the optimal concentrations for each oligo to be 10–25 nM in a 50 μL reaction, and that the amplification primers must be at least tenfold more concentrated than the assembly oligos (11). They used 0.4 μM amplification primers, which represents a 16–40-fold excess of primer to internal oligos.
21. Alternately, Prodromou and Pearl suggest 30 cycles of 2 min at 95°C (denaturation), 2 min at 56°C (annealing), and 1 min at 72°C (extension), with a final extension at 72°C for 10 min when using Vent DNA polymerase (7). Freeman suggests additional temperature cycle options based on the performance of their preferred DNA polymerases *KOD* XL and *KOD* Hifi (11).

22. 1× TBE buffer (89 mM Tris base, 89 mM boric acid, and 2 mM EDTA at pH 8.0) may be used as a substitute for 1× TAE in agarose preparation and running buffer to improve resolution for target products below 250 bp. 10× TBE can be prepared by adding 108 g of Tris base, 55 g of boric acid, and 20 mL of 0.5 M EDTA to 1 L of ultrapure water. Dilute tenfold in ultrapure water to obtain 1× TBE. An alternate ladder with 25 bp steps, such as Bioline's Hyperladder V, can be used for molecular weight comparison when working with smaller DNA targets.
23. Optionally, add ethidium bromide to dissolved agarose or electrophoresis running buffer to a final concentration of 0.5 μg/mL.
24. If bubbles appear in the agarose upon mixing, either pierce them with a pipette tip or move them to the sides of the casting tray with the pipette to prevent interference with DNA migration.
25. A 5–10 μL total volume is appropriate for a diagnostic gel using an 8–12-well comb. A 20 μL total volume (marker) to 55 μL total volume (PCR) is appropriate for preparative gels using a 1.5 mm thick 6-well comb.
26. Note that a 1.5 mm thick 6-well comb should be selected for this gel to maximize well volume. Use of this comb may be best accommodated by adding an additional 25–50% mass of molecular weight ladder and increasing the total volume of ladder and dye loaded to the gel to 20 μL.
27. If the target band is not easily identifiable on the preparative gel, choose multiple candidate bands and excise them separately, noting the position of each excised slice on a copy of the gel image. Purify DNA from the gel slice as described in subsequent steps, and test for the presence of the target sequence by secondary PCR before proceeding with digestion and ligation (see Note 29).
28. Optionally, Prodromou used a sequential extraction with phenol, phenol/chloroform, chloroform and ether, and finally ethanol precipitation to purify the target product from the agarose (7).
29. The presence or absence of the target DNA in a given sample can be assessed by secondary PCR, which in this case is a subsequent amplification of the preparative gel-purified DNA. Assemble the secondary PCR using the same recipe as given in Table 1, replacing the DNA oligo mix with 50–100 ng of the gel-purified DNA. Use the same temperature cycling conditions used for the original PCR (e.g., if the sample was obtained from an R-PCR with a 59°C annealing temperature, use 59°C for the annealing step in the secondary PCR as well). Analyze the amplification product on an analytical agarose gel as described

(see Subheading 3.2, steps 4–9). A secondary PCR amplification product corresponding to the target DNA base pair length suggests that the original R-PCR and preparative gel DNA extraction were successful.

30. Prodromou (7) successfully digested with both *Eco*RI and *Hind*III in the same reaction; however other literature recommends the sequential digest described here for optimal cleavage. The enzyme manufacturer's literature should be consulted to determine whether a mutually compatible buffer exists for the selected pair of enzymes. If so, perform a double digest in the compatible buffer by adding 0.5 μL of both enzymes to the digestion reaction in Subheading 3.4, step 1, incubating at 37°C for 1 h, and then proceeding directly to Subheading 3.4, step 4.
31. Instead of a spin column kit, Prodromou (7) used Strataclean resin (Stratagene, Cambridge, UK) to purify the DNA from the restriction endonucleases.
32. For some DNA templates, a 15-min Quick Ligase™ reaction does not reliably yield colonies upon transformation. In such circumstance, a >16-h ligation at room temperature with T4 DNA Ligase and Buffer (New England Biolabs) is generally successful. The addition of 5–10% v/v DMSO can increase the ligation efficiency of templates with difficult secondary structure regions, but is accompanied by a decrease in transformation efficiency. Quick Ligase™ is not appropriate for extended ligations due to the presence of polyethylene glycol in the ligation buffer.
33. If using a compatible vector, optional blue/white colony screening can be performed by plating onto LB Amp plates containing X-gal (20 μg/mL) and IPTG (32 μg/mL). Colonies that appear white should have an insert ligated into the vector, while colonies that appear blue do not.
34. Freeland performed an exhaustive study on the error rates of *KOD* XL and *KOD* Hifi DNA polymerases, and formulated equations for colony screening that account for the probability of finding an insert with an exact sequence (11). These formulas are based on the length of the target product and the error rate of each polymerase.
35. DNA pelleted in isopropanol forms a glassy, translucent pellet that can be difficult to visualize. Using clear conical-bottomed centrifuge tubes and marking the tube before centrifugation may help in visualizing the pellet to ensure that it is not lost. Isopropanol pellets also do not stick to the side of centrifuge tubes well, so be very careful when drawing off the supernatant and subsequently washing the pellet.

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