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Implementing Novel Designs in pET Expression Plasmids that Increase Protein Production

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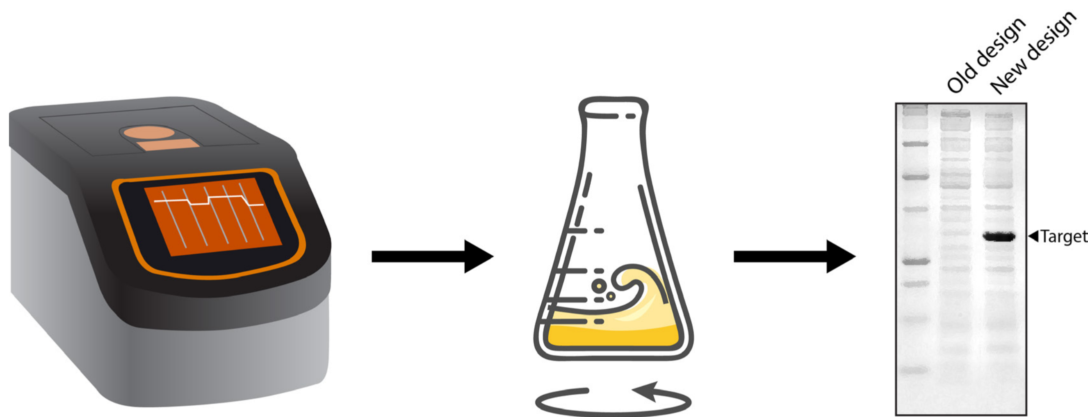
[Abstract] pET expression plasmids are widely used in the biotechnology, biopharmaceutical, and basic research sectors for the production of recombinant proteins. Typically, they are used off-the-shelf because they support high production titers; however, we have identified two design flaws in many pET plasmids that limit their production capacity. We used modern methods of DNA assembly and directed evolution to identify improved designs for these modules and demonstrated that these designs support higher protein production yields. Herein, we present two PCR protocols for implementing the designs and increasing protein production from existing pET expression plasmids.

Graphic abstract:



A simple workflow for implementing novel designs in pET expression plasmids.

Keywords: pET, Plasmid, T7*lac*, Transcription initiation, Translation initiation region (TIR), Synthetic evolution, Bacterial cell factory, Recombinant protein

[Background] The basic architecture of pET expression plasmids was established over three decades ago by integrating the φ10 promoter for the T7 RNA polymerase (*T7p*) and the Tφ transcription terminator (*T7t*) into the pBR322 backbone (Rosenberg *et al.*, 1987). This architecture enables efficient transcription of cloned coding sequences in bacterial strains harbouring an inducible copy of the *DE3* phage fragment encoding the T7 RNA polymerase. The basic pET vector architecture was then elaborated on by including optional add-ons. For example, a Shine-Dalgarno (SD) sequence originating

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from the major capsid protein of T7 (gene 10 protein) was incorporated to enable efficient translation initiation (Rosenberg *et al*., 1987), and the *lac* O_1 operator sequence was cloned adjacent to the T7 promoter (*T7lac*) so that basal gene expression was repressed in the absence of an inducer (Dubendorf and Studier, 1991). Alternative antibiotic cassettes and purification, solubility, and secretion tags were also included. Currently, 103 different pET expression plasmids are available (Shilling *et al.* 2020). It is also possible to construct bespoke T7p-based expression plasmids using the modular platform for Standard European Vector Architecture (SEVA) (Silva-Rocha *et al*., 2013).

pET expression plasmids are currently used 'off-the-shelf' because they are known to support high titers of recombinant protein production - as much as 50% of the total cell protein after a few hours of induction (Mierendorf *et al*., 1998). Titers can also be increased by screening induction conditions or testing bacterial hosts that are supplemented with tRNAs and folding catalysts or that modulate the expression of T7 RNA polymerase (Rosano and Ceccarelli, 2014; Rosano *et al*., 2019). However, not all recombinant proteins can be expressed at high titers and many fall out of experimental pipelines. For example, analysis of structural genomics pipelines indicated that more than 40% of soluble proteins cannot be produced in sufficient titers for downstream structural, biochemical, and biophysical studies (Walsh, 2015; Parret *et al*., 2016).

In a recent study, we identified a design flaw in the *T7lac* module (Shilling *et al*., 2020). This module was originally engineered by fusing *T7p* to the *lac* O_1 operator sequence in the early generation pET plasmids (Dubendorf and Studier, 1991). *T7p* is typically 23 nucleotides long and sits −17 to +6 relative to the messenger RNA (mRNA) start site (Figure 1) (Dunn *et al*., 1983). However, it was truncated by four nucleotides when *lac* O_1 was fused, as the architects used an *Stu*I restriction site within *T7p*. In our previous study, we used an overlap PCR approach to insert the four truncated nucleotides into *T7lac* in the most commonly used pET expression plasmid, pET28a(+). We subsequently demonstrated that this design (*T7p^CONS^lac*) increased the production titers of recombinant proteins (Shilling *et al*., 2020). Herein, we present a protocol for incorporating the *T7p^CONS^lac* design in vectors with an existing *T7lac* module (Protocol 1). This protocol is directly applicable to 88 different pET plasmids (Table 1), as well as the +LacIq-PT7/LacO (SEVA#4E) module of the pSEVA platform (Silva-Rocha *et al*., 2013). The remaining 15 pET vectors encode *T7p^CONS^* and do not include the *lac* O_1 operator sequence.

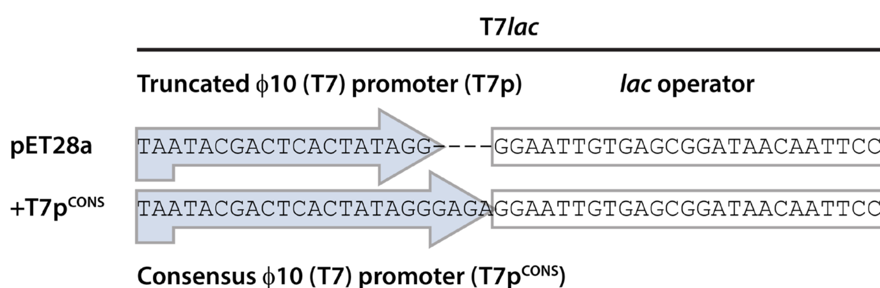

Figure 1. Comparison of *T7* promoters. The *T7* promoter in pET28a(+) and 87 other pET plasmids is a truncated variant fused to the *lac* operator. Protocol 1 uses an overlap PCR approach to insert four nucleotides into the *T7* promoter, thus restoring the consensus sequence and increasing production titers. Figure adapted from Shilling *et al*. (2020).

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Our recent study also identified a design flaw in the translation initiation region (*TIR*) of pET28a(+). This module is a stretch of 30 nucleotides that is recognised by the 30S subunit of the ribosome during translation initiation (*i.e.*, the first ribosomal footprint). The sequence determines the efficiency of translation initiation, the rate-limiting step in protein synthesis (McCarthy and Gualerzi, 1990; Laursen *et al*., 2005; Milón and Rodnina, 2012), and significantly affects the production titers of recombinant proteins (Mirzadeh *et al*., 2015, 2016 and 2020; Shilling *et al*., 2020). The sequence is usually comprised of: (1) a Shine-Dalgarno sequence complementary to the 16S rRNA subunit; (2) an AUG start codon that is situated 5-9 nucleotides downstream; and (3) the first 5 codons of the coding sequence (Shine and Dalgarno, 1975; Chen *et al*., 1994; Osterman *et al*., 2013). A large body of work indicates that the *TIR* works most effectively when it is largely free of mRNA structures, which promotes accessibility of the 30S subunit (Kudla *et al*., 2009; Plotkin and Kudla, 2011; Bentele *et al*., 2013; Goodman *et al*., 2013). In most pET expression plasmids, this region is comprised of the SD sequence and a seven-nucleotide spacer region from the major capsid protein of T7, and the first five codons of the coding sequence. However, there is no indication that this region has been optimised in any pET expression plasmid, which we considered a design flaw. We therefore carried out a directed evolution approach on the *TIR* in pET28a(+), which encodes an N-terminal poly-histidine tag and a thrombin protease cleavage site (Figure 2). We identified two *TIRs* that work more efficiently than the existing *TIR*, as judged by the fact that they increase protein production titers (Shilling *et al*., 2020). Herein, we present a protocol for incorporating the improved *TIRs* (Protocol 2), which is directly applicable to four of the most widely used pET plasmids (Table 1). Utilising the optimisation strategy, we noted improvements to sfGFP expression levels, starting from a low of 0.8 mg/ml to a high of 97 mg/ml, without affecting protein quality (Shilling *et al*., 2020). In instances where protein expression was already determined to be high, the addition of optimised *TIRs* described in this protocol did not always result in an increase in protein yield (unpublished data).

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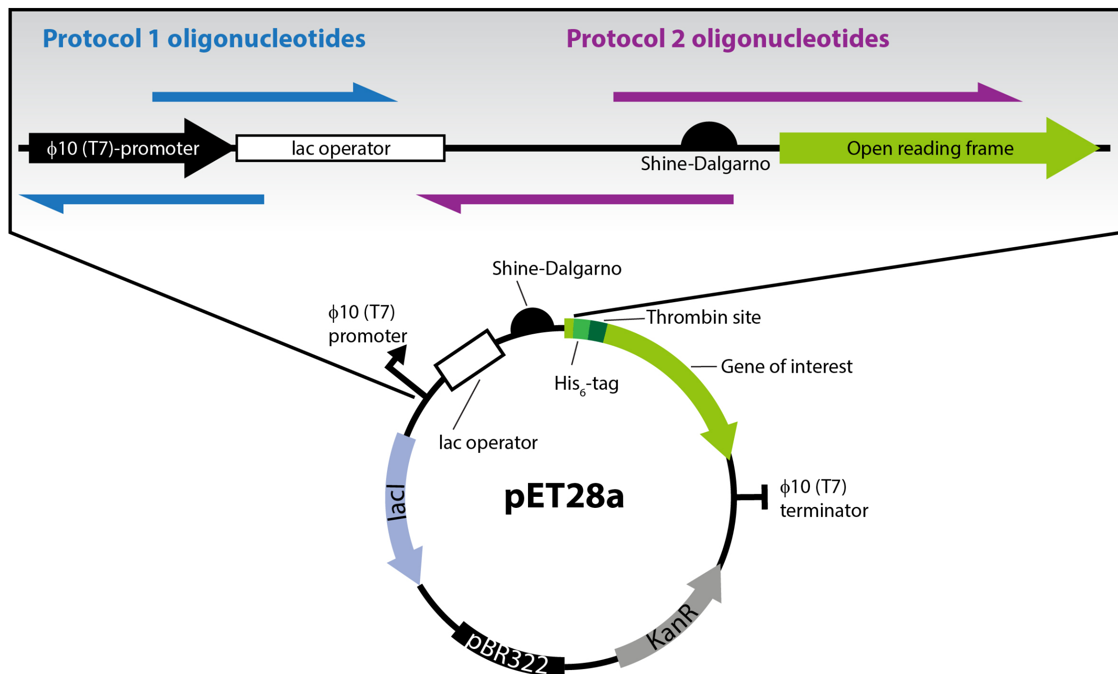

Figure 2. Features of the pET28a(+) plasmid and position of oligonucleotides used in the two PCR protocols. The pET28a plasmid includes the φ10 (T7) promoter and the lac operator, as well as the translation initiation region (*TIR*) encompassing the Shine-Dalgarno (SD) sequence, a spacer, and the first five codons of the open reading frame. Protocol 1 oligonucleotides (blue) incorporate four nucleotides within the T7 promoter at +3-6 relative to the mRNA transcriptional start site. Protocol 2 oligonucleotides (purple) incorporate nucleotide mutations in the *TIR*, which increases protein production titers.

Materials and Reagents

1. Petri dishes (VWR, catalog number: 391-0440)
2. Plate culture spreaders (VWR, catalog number: 612-1561)
3. Inoculation loops (VWR, catalog number: 612-9352)
4. 50-ml conical tubes (VWR, catalog number: 525-0402)
5. Chemically competent *E. coli* MC1061
6. Q5 polymerase (NEB, catalog number: M0491L, storage: -20°C)
7. *Dpn*I (NEB, catalog number: R0176L, storage: -20°C)
8. dNTPs (Thermo Scientific, catalog number: R0181, storage: -20°C)
9. Oligonucleotides (Eurofins Genomics)
10. Agarose (Sigma-Aldrich, catalog number: A9539)
11. O'GeneRuler DNA Ladder (Thermo Scientific, catalog number: SM1163)
12. DNA miniprep kit (OmegaTek, catalog number: D6943-02)
13. Yeast extract (Oxoid, catalog number: LP0021)
14. Tryptone (Oxoid, catalog number: LP0042)

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15. NaCl (VWR, catalog number: ICNA0219473805)
16. Agar (VWR, catalog number: 20767.298)
17. Kanamycin sulfate (VWR, catalog number: 0408-EU-25G, storage: 4°C)
18. Ampicillin sodium salt (Sigma, catalog number: A0166-25G, storage: 4°C)
19. LB medium (see Recipes)
20. Antibiotic stock solutions (see Recipes)
21. LB agar (see Recipes)
22. 50× TAE buffer (see Recipes)
23. DNA loading buffer (see Recipes)

Equipment

1. Thermocycler (Techne, catalog number: 5PRIME/02)
2. DNA mini horizontal submarine unit (Hoefer, catalog number: HE33)
3. Electrophoresis power supply (GE Healthcare, catalog number: EPS601)
4. Gel Imager Azure C200 (Azure Biosystems, catalog number: AC2001)
5. Thermomixer Comfort (Eppendorf, catalog number: 5355000.011)
6. New Brunswick Incubator (New Brunswick, catalog number: M1282-0012)
7. Benchtop centrifuge (Eppendorf, model: 5417C)
8. NanoDrop Spectrophotometer (Thermo Scientific, catalog number: ND-2000)

Procedure

A. Check whether the protocols are relevant to the pET plasmid of interest by cross-referencing Tables 1 and 2. If so, order the required oligonucleotide set.
    1. Protocol 1 will correct a design flaw in the T7*lac* module, converting it to T7p^{CONS}*lac.* This protocol only applies to the pET plasmids listed in Table 1.
    2. Protocol 2 will replace the standard TIR with one of two optimised TIRs. This protocol only applies to pET expression plasmids encoding an N-terminal poly-histidine tag, such as those listed in Table 1.

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Table 1. pET plasmids that will benefit from each protocol

Protocol 1 (implementation of T7p^{CONS}*lac*)				
pET11a-d	pET31b	pET46Ek/LIC	pET-SUMO/CAT	pET160/D-TOPO
pET15b	pET32a-c	pET47b	pET100/D-TOPO	pET160/GW/CAT
pET16b	pET32Ek/LIC	pET48b	pET100/D/LacZ	pET161/D-TOPO
pET19b	pET32Xa/LIC	pET49b	pET101/D-TOPO	pET161-DEST
pET21a-d	pET33b	pET50b	pET101/D/LacZ	pET161-GW/CAT
pET22b	pET39b	pET51b	pET102/D-TOPO	pET200/D-TOPO
pET24a-d	pET40b	pET51Ek/LIC	pET102/D/LacZ	pET200/D/LacZ
pET25b	pET41a-c	pET52b	pET104-DEST	pET300/NT-DEST
pET26b	pET41Ek/LIC	pET52-3C/LIC	pET104/GW/LacZ	pET300/NT-GW/Rac Kinase
pET27b	pET42a-c	pETDuet-1	pET104.1-DEST	pET301/CT-DEST
pET28a-c	pET43.1a-c	pET-Dest41	pET104.1/GW/LacZ	pET302/NT-DEST
pET29a-c	pET44a-c	pET-Dest42	pET151/D-TOPO	pET303/CT-DEST
pET30a	pET45b	pET-SUMO	pET151/D/LacZ	pET303/CT-His Rac Kinase
Protocol 2 (implementation of novel TIRs)				
pET14b	pET15b	pET28a-c	pET33b	

3. Order the oligonucleotide sets required for implementing the T7^{CONS}*lac* promoter (Protocol 1) and/or the TIR (Protocol 2). The oligonucleotides have complementarity to the plasmid template at their 3' ends and to each other at their 5' ends. The sequences are provided in Table 2.

Table 2. Oligonucleotide sequences for correction of design flaws

Primer set	Number	Sense	Primer sequence (5'-3')
Protocol 1	1	Forward	CTATAGGGAGAGGAATTGTGAGCGGATAAC
	2	Reverse	CCTCTCCCTATAGTGAGTCGTATTAATTTCGCGG
Protocol 2	3	Forward	AACTTTAAGAAGGAGAGTTATCATGGGTAGCAGCCATCATCATCATCATCA
	4	Forward	AACTTTAAGAAGGAGAGCAGCTATGCAGCTTAGCCATCATCATCATCATCA
	5	Reverse	CTCCTTCTTAAAGTTAAACAAAATTATTTCTAGAGGGGAATTGTTATC

B. PCR setup and program for Protocol 1

1. Combine the following reagents (Final reaction volume 25 µl):
    a. 5 µl Q5 reaction buffer
    b. 5 µl GC enhancer solution (optional: included with the Q5 polymerase kit)
    c. 1 µl dNTPs from a 10 mM stock (0.4 mM final concentration)
    d. 1.25 µl primer 1 from a 10 µM stock (0.5 µM final concentration)
    e. 1.25 µl primer 2 from a 10 µM stock (0.5 µM final concentration)

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f. 0.5 µl template plasmid from a 2 ng/µl stock (0.04 ng final concentration)
g. 0.25 µl Q5 polymerase
h. 10.75 µl sterile ultrapure water

2. Set up a PCR program with the following parameters:
   a. Hold at 95°C for 5 min
   b. First cycle with 5 repeats
      95°C for 30 s
      48°C for 30 s
      72°C for 3.5 min
   c. Second cycle with 20 repeats
      95°C for 30 s
      60°C for 30 s
      72°C for 3.5 min
   d. Infinite hold at 10°C

C. PCR setup and program for Protocol 2

1. Combine the following reagents (*TIR-1* or *TIR-2*) (Final reaction volume 25 µl):
   a. 5 µl Q5 reaction buffer
   b. 5 µl GC enhancer solution (optional)
   c. 1 µl dNTPs from a 10 mM stock (0.4 mM final concentration)
   d. 1.25 µl primer 3 (TIR-1) or 4 from a 10 µM stock (TIR-2) (0.5 µM final concentration)
   e. 1.25 µl primer 5 from a 10 µM stock (0.5 µM final concentration)
   f. 0.5 µl template plasmid from a 2 ng/µl stock (2 ng final concentration)
   g. 0.25 µl Q5 polymerase
   h. 10.75 µl sterile ultrapure water
2. Set up a PCR program with the following parameters:
   a. Hold at 95°C for 5 min
   b. First cycle with 5 repeats
      95°C for 30 s
      48°C for 30 s
      72°C for 3.5 min
   c. Second cycle with 20 repeats
      95°C for 30 s
      60°C for 30 s
      72°C for 3.5 min
   d. Infinite hold at 10°C

D. Check the PCR product by agarose gel electrophoresis

1. Cast a 1% (w/v) agarose gel with 1× TAE buffer and an appropriate gel stain.

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2. Mix 3 µl PCR reaction with a suitable DNA loading buffer.
3. Load the samples and perform electrophoresis at a constant 100 V for 30 min.
4. Visualise the gel on a suitable imaging workstation (such as the Azure 200, Azure Biosystems). An example of an expected PCR product is shown in Figure 3.

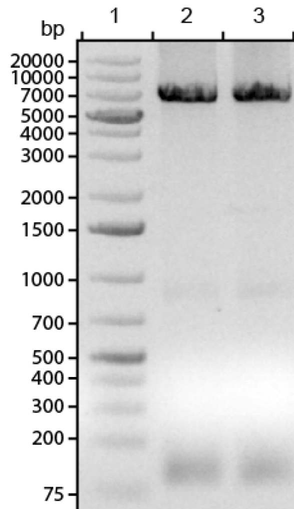

Figure 3. Example of an expected PCR product for protocol 2. A 1% agarose gel in TAE buffer. Lane 1: O'GeneRuler 1-kb Plus DNA Ladder (Thermo Scientific). Lane 2: PCR product using primers 3 and 5. Lane 3: PCR product using primers 4 and 5.

E. Perform *Dpn*I treatment of the PCR product
   1. Combine the following in a 200-µl PCR tube:
      a. 4 µl PCR product (Optional: Perform PCR clean-up of the sample prior to *Dpn*I treatment)
      b. 0.5 µl Cutsmart buffer (included in the *Dpn*I kit)
      c. 0.5 µl *Dpn*I
   2. Incubate the samples at 37°C for 1 h in a thermocycler (such as the Techne Large-Format Gradient Thermal Cycler).
   3. Optional: Heat inactivate *Dpn*I at 80°C for 15 min.

F. Transform MC1061 with the *Dpn*I-treated PCR product
   1. Add 5 µl *Dpn*I-treated sample to 50 µl chemically competent *E. coli* such as MC1061 or an alternative strain capable of *in vivo* DNA assembly.
   2. Incubate on ice for 30 min.
   3. Heat shock at 42°C for 1 min.
   4. Incubate on ice for 2 min.
   5. Add 150 µl LB medium.
   6. Incubate at 37°C for 30 min with shaking at 900 rpm using a thermomixer (such as the Eppendorf Thermomixer R).
   7. Plate 100 µl cell culture onto LB-agar containing a suitable antibiotic.

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8. Allow the plate to dry in an aseptic environment.
9. Incubate the plate face-down overnight at 37°C.

G. Pick colonies and sequence

1. Pick two individually separated colonies and inoculate 10 ml LB media with a suitable antibiotic in a 50-ml conical tube.
2. Incubate the inoculated cultures at 37°C overnight with shaking at 180 rpm.
3. Harvest the cultures by centrifugation at 3,220 × *g* for 10 min.
4. Extract the plasmids using a miniprep kit such as the E.Z.N.A DNA mini kit from Omega Bio-Tek.
5. Measure the DNA concentration using a spectrophotometer such as the NanoDrop 2000.
6. Send the purified plasmids for sequencing.
   Note: Use appropriate primers that are a minimum of 60 bp from the site of mutagenesis.
7. Confirm insertion of the new modified DNA sequence by comparison with the expected sequence using a standalone program such as SnapGene (download from https://www.snapgene.com/) or an online browser-based program such as Benchling (access from https://www.benchling.com/).

Recipes

1. LB medium
   10 g/L NaCl
   10 g/L tryptone
   5 g/L yeast extract
   Dissolve in 1 L ultrapure water and sterilise by autoclaving
2. Antibiotic stock solutions
   Weigh the required quantity of antibiotics; 50 mg/ml kanamycin or 100 mg/ml ampicillin.
   Add ultrapure water to the desired volume and dissolve by vortexing.
   Under aseptic conditions, filter sterilise the antibiotic solution into the appropriate volumes.
   Use directly or store at -20°C.
3. LB agar
   LB medium plus 15 g/L agar
   Dissolve in 1 L ultrapure water and sterilise by autoclaving.
   Allow the media to cool to ~50°C.
   Add the appropriate antibiotic and pour 20-ml volumes into 9.5-cm Petri dishes.
4. Tris acetic acid (TAE) (50×)
   242.2 g/L Tris base (2 M)
   57.1 ml/L acetic acid (1 M)
   18.6 g/L EDTA (50 mM)
5. 6× DNA loading buffer

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60% v/v glycerol
20 mM Tris pH 8.0
60 mM EDTA
0.03% bromophenol blue

Acknowledgments

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Competing interests

The protocols and designs described herein are free from intellectual property. However, the synthetic evolution process used to identify *TIR-1* and *TIR-2* is patent-protected (PCT/SE2015/051343; European Patent no. 3234146). These patents are the property of CloneOpt AB, of which P.J.S. is a former employee and D.O.D. is a shareholder.

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