RTS™ 100 *E. coli* LinTempGenSet, His-tag Manual

For rapid production of linear expression templates using PCR

RTS 100 *E. coli* LinTempGenSet, His-tag Manual, April, 2015

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Product specifications

The RTS 100 *E. coli* LinTempGenSet, His-tag is a module to rapidly generate linear expression constructs by PCR. The constructs are ready for use as DNA templates in RTS 100 *E. coli* HY *in vitro* protein expression reactions without the need for purification.

Product description

The RTS 100 *E. coli* LinTempGenSet, His-tag provides the components and procedures necessary for generation of linear expression constructs by PCR.

Product limitations

RTS 100 *E. coli* LinTempGenSet, His-tag is developed, designed, and sold for research purposes only. It is not to be used for human diagnostic or drug purposes or to be administered to humans unless expressly cleared for that purpose by the Food and Drug Administration in the USA or the appropriate regulatory authorities in the country of use. All due care and attention should be exercised in the handling of the materials described in this text.

Materials supplied

Kit	RTS 100 *E. coli* LinTempGenSet, His-tag
Ordering number	BR1400801
T7 Promoter Primer; E.coli LTGS	4 vials, 5× conc. (24 reactions each)
T7 Terminator Primer; E.coli LTGS	4 vials, 5× conc. (24 reactions each)
C-terminal His_6-tag DNA; E.coli LTGS	1 vial (96 reactions)
N-terminal His_6-tag DNA; E.coli LTGS	1 vial (96 reactions)
RNase-DNase-free Water; E.coli LTGS	2 vials (1 ml each)

Additional materials

To perform the protocols described in this manual, the following additional materials must be provided by the user:

- Expand™ High Fidelity PCR System (Roche)

- PCR Nucleotide Mix DNA (Roche)
- Molecular Weight Marker VII for PCR fragments of more than 1 kb (Roche)
- DNA Molecular Weight Marker VIII for fragments up to 1 kb (Roche)
- Agarose MP (Roche)
- Gene-specific primers (see Protocol 1, page 9)

For convenience, additional materials to be supplied by the user are listed at the beginning of the protocol for which they are required.

Shipping and storage conditions

The RTS 100 *E. coli* LinTempGenSet, His-tag is shipped on dry ice.

RTS 100 *E. coli* LinTempGenSet, His-tag should be stored at (–15 to –25°C) and is stable until the expiration date printed on the label. Avoid repeated freezing and thawing.

Safety information

All due care and attention should be exercised in the handling of this product. We recommend all users of biotechrabbit products to adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines. Specifically, always wear a suitable lab coat, disposable gloves, and protective goggles when working with chemicals.

Quality assurance

biotechrabbit products are manufactured using quality chemicals and materials that meet our high standards. All product components are subjected to rigorous quality assurance testing process:

- **Component testing:** each component is tested to ensure the composition and quality meet stated specifications.
- **Performance testing:** each product is tested to ensure it meets the stated performance specification.

Additional quality information is available from www.biotechrabbit.com. Certificates of Analysis are available on request.

Product warranty

biotechrabbit is committed to providing products that improve the speed, ease-of-use and quality of enabling technologies.

biotechrabbit guarantees the performance of all products in the manner described in our product literature. The purchaser must determine the suitability of the product for its particular use.

This warranty is in place of any other warranty or guarantee, expressed or implied, instituted by law or otherwise. biotechrabbit provides no other warranties of any kind,

expressed or implied, including warranties of merchantability and fitness for a particular purpose. Under no circumstance shall biotechrabbit be responsible for any direct, indirect, consequential or incidental damages or loss arising from the use, misuse, results of use or inability to use its products, even if the possibility of such loss, damage or expense was known by biotechrabbit.

Protocols

Product principle

Introduction

The RTS 100 *E. coli* LinTempGenSets are designed for the rapid and convenient production of linear expression templates. The procedure does not involve cloning and the optimized expression cassette allows flexible positioning of the His_6-tag. The templates can be subsequently used for rapid *in vitro* protein expression using the RTS 100 *E. coli* HY Kit.

In order to generate linear expression constructs, two PCR reactions must be performed. In the first reaction, gene-specific primers are used to add overlap regions to the sequence of the target gene. The product of the first PCR is mixed with two flanking primers and the DNA fragments coding for the T7 regulatory elements and a His_6-tag sequence. In the second, overlap extension PCR, the product of the first PCR anneals with the added DNA fragments and the 3' ends are extended. This linear expression construct is finally amplified via the flanking primers (Figure 1).

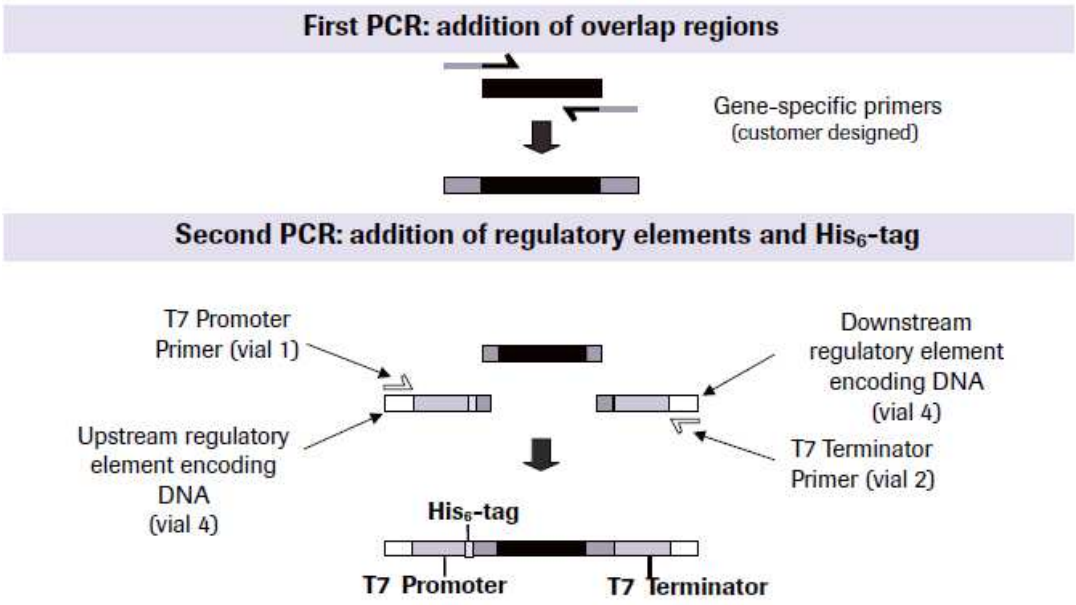

Figure 1. Principle of the generation of PCR products for *in vitro* protein expression via the RTS 100 *E. coli* LinTempGenSet, His-tag.

As an example, the introduction of an N-terminal His_6-tag is shown.

Description of procedure

The RTS workflow (Figure 2) combines a series of technologies for efficient and optimized protein expression. They overcome the limitations that often restrict the use of cell-free systems. These innovations include software-based template optimization, generation of stable expression templates without cloning, optimization of *in vitro* expression conditions, high yield *in vitro* expression using the continuous exchange cell-free principle (CECF), and an optimized lysate biochemistry.

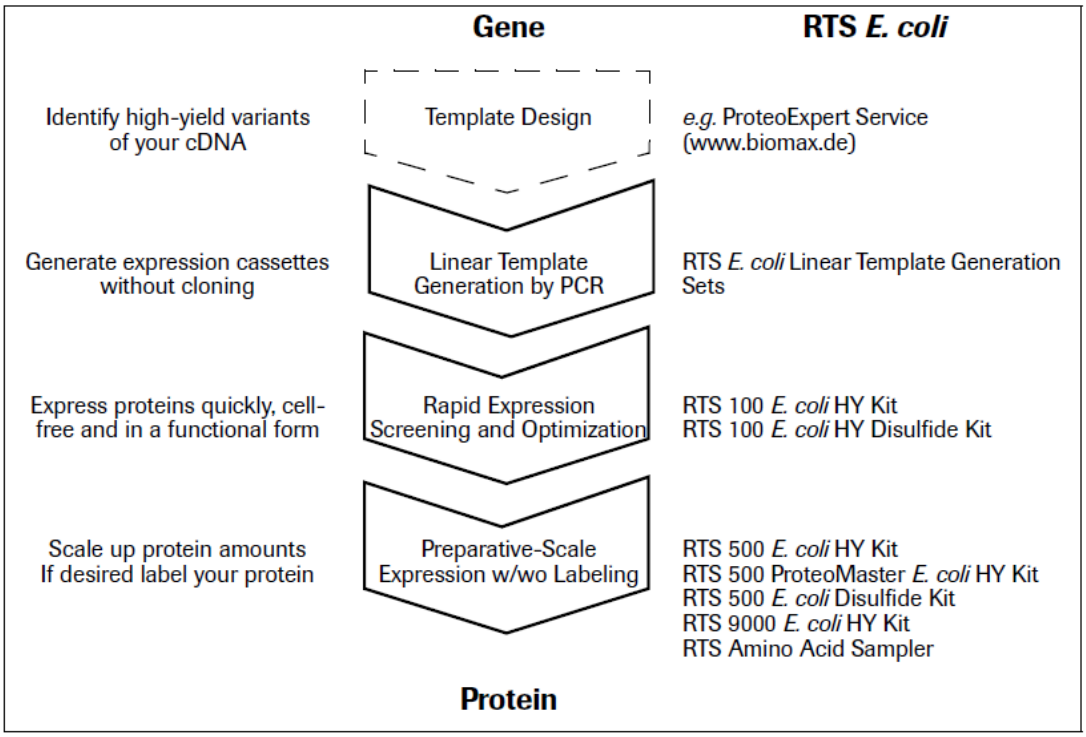

Figure 2. Integration of the RTS 100 *E. coli* LinTempGenSets in the overall RTS workflow.

Protocol 1: Design of gene-specific primers for first PCR

Before starting

- The nucleotide sequences of the primers and DNA provided with this kit can be downloaded from www.biotechrabbit.com
- As gene-specific primers biotechrabbit recommends standard-quality primers in a synthesis scale of 0.02–0.04 µmol
- The T_m for both gene-specific primers should be similar
- For information on primer design see references, page 17

Procedure

1. Design and order gene-specific primers for the desired tag position for the first PCR according to Table 1.

Table 1. Primers

	Gene-specific sense primer	Gene-specific antisense primer
C-terminal His_6-tag	5'-CTTTAAGAAGGAGATATACC +ATG +15–20 nt specific for the gene sequence to be expressed (i.e. beginning with ATG)	5'-TGATGATGAGAACCCCCCCC +15 –20 nt specific for the gene sequence to be expressed (ensuring that no stop codon from the gene-specific sequence inhibits tag expression)
N-terminal His_6-tag	5'-CGCTTAATTAAACATATGACC +15–20 nt specific for the gene sequence to be expressed (no ATG necessary)	5'-TTAGTTAGTTACCGGATCCC +TTA +15–20 nt specific for the gene sequence to be expressed
No tag	5'-CTTTAAGAAGGAGATATACC +ATG +15–20 nt specific for the gene sequence to be expressed (i.e. beginning with ATG)	5´- TGATGATGAGAACCCCCCCC +TTA +15–20 nt specific for the gene sequence to be expressed

Protocol 2: Generation of linear fragments for *in vitro* expression

Before starting

- The amount of template DNA should be:
  - up to 500 ng of genomic DNA
  - up to 250 ng of plasmid DNA
- When starting with a plasmid containing a T7 promoter and a T7 terminator sequence (e.g. pIVEX, pET, or pDEST™ vectors) the first PCR product must be eluted from an agarose gel using the GenUP™ Gel Extraction Kit (cat. no. BR0700401, biotechrabbit) to prevent contamination of the linear template with the plasmid.

Procedure

First PCR: Addition of overlap regions

1. Set up the first PCR according to Table 2.

   Use the Expand High Fidelity PCR System and the PCR Nucleotide Mix (Roche) for the PCR.

Table 2. First PCR components

Component	Volume	Final concentration
Expand High Fidelity buffer, 10× conc. without $MgCl_2$	5 µl	1x
$MgCl_2$, stock solution (25 mM)	3–6 µl	1.5–3.0 mM
PCR Nucleotide Mix	1.25 µl	250 µM
Gene-specific sense primer	x µl	101–300 nM
Gene-specific antisense primer	y µl	101–300 nM
Expand High Fidelity Enzyme mix	0.85 µl	3U
Template DNA	z µl	1–500 ng
PCR grade H_2O	Up to 50 µl	

2. Set up the cycle profile for the thermocycler according to Table 3.

   Note: The cycle profile given is for the ABI GeneAmp® 9600 Thermocycler. When using other thermocyclers, the cycle conditions must be adjusted.

Table 3. First PCR cycle profile

Cycles	Time	Temperature, °C	Remarks
1	4 min	94	Denaturation
20	1 min	94	Denaturation
	1 min	45–60*	Annealing
	45 s – 2 min†	72	Elongation
1	Up to 7 min	72	Elongation

* Annealing temperature depends on the melting temperature of the primer used. Formula for melting point (T_m) calculation: T_m = (number of A + T) × 2°C + (number of G + C) × 4°C; annealing temperature = T_m – 5°C.

† Elongation time depends on fragment length. biotechrabbit recommends 1 min for 1.0 kb.

Second PCR: Addition of regulatory elements and His_6-tag

Procedure

1. Prepare the working solutions according to steps 2–5.
2. Add 80 µl PCR-grade H_2O (vial 5) to T7 Promoter Primer (vial 1) for a final concentration of 6 µM.
3. Add 80 µl PCR-grade H_2O (vial 5) to T7 Terminator Primer (vial 2) for a final concentration of 6 µM.
4. Add 90 µl PCR-grade H_2O (vial 5) to C-terminal His_6-tag DNA (vial 3) for a final concentration of 50x.

   Aliquot into small quantities to avoid repeated freeze–thaw cycles (10–20 µl, depending on the number of reactions performed in one PCR set up).
5. Add 90 µl PCR-grade H_2O (vial 5) to N-terminal His_6-tag DNA (vial 4) for a final concentration of 50x.

   Aliquot into small quantities to avoid repeated freeze–thaw cycles (10–20 µl, depending on the number of reactions performed in one PCR set up).
6. Analyze the product of the first PCR on a 0.8–1.5% agarose gel. The product of the first PCR must be a dominant band of at least 80% purity.

   See Troubleshooting, page 18 for optimization tips.

   If nonspecific byproducts are observed after adapting the PCR conditions, elute the specific band from an agarose gel using the GenUP™ Gel Extraction Kit (biotechrabbit).
7. Use 2–4 µl (approx. 150–300 ng) of the product of the first PCR as template for the second PCR (for quantification, see Protocol 3: Quantification of PCR product for expression with the RTS 100 *E. coli* HY Kit, page 13).

   The PCR conditions and the cycle profile for templates longer than 2.5–3.0 kb must be optimized. The following protocols are optimized for templates smaller than 3 kb.

8. Set up the second PCR according to Table 4.

   Use the Expand High Fidelity PCR System and the PCR Nucleotide Mix (Roche) for the PCR.

Table 4. Second PCR components

Component	Volume	Final concentration
Expand High Fidelity buffer, 10× conc. without $MgCl_2$	5 µl	1x
$MgCl_2$, stock solution (25 mM)	x µl	1.5–3.0 mM
PCR Nucleotide Mix	1.25 µl	250 µM each
Vial 1 working solution: T7 Promoter Primer (6 µM)	4 µl	480 nM
Vial 2 working solution: T7 Terminator Primer (6 µM)	4 µl	480 nM
Vial 3 **or** 4 working solution: C-terminal **or** N-terminal His_6-tag DNA. For native protein expression without tag, use vial 3 working solution	1 µl	1x
Expand High Fidelity Enzyme mix	0.85 µl	3U
Template: Product of the first PCR	y µl	150–300 ng
PCR grade H_2O		Up to 50 µl

9. Set up the cycle profile for the thermocycler according to Table 5.

   Note: The cycle profile given is for the ABI GeneAmp 9600 Thermocycler. When using other thermocyclers, the cycle conditions must be adjusted.

Table 5. Second PCR cycle profile

Cycles	Time	Temperature, °C	Remarks
1	4 min	94	Denaturation
20–25	1 min	94	Denaturation
	1 min	60	Annealing
	≥1 min*	72	Elongation
1	Up to 7 min	72	Elongation

* Elongation time depends on fragment length. biotechrabbit recommends 1 min for 1.0 kb plus 0.5 min for the amplification of the additional regulatory sequences.

Protocol 3: Quantification of PCR product for expression with the RTS 100 *E. coli* HY Kit

Procedure

1. Run 0.5 µg and 1 µg DNA molecular weight marker as well as 1 µl and 2 µl product of the first and second PCR on a 1.0–1.5% agarose gel.
2. Estimate the product of the second PCR using the content of the molecular weight marker bands as an internal standard (Figure 3).
3. Use 50–150 ng product of the second PCR for expression in a 50 µl reaction volume with RTS 100 *E. coli* HY Kit (Figure 4).

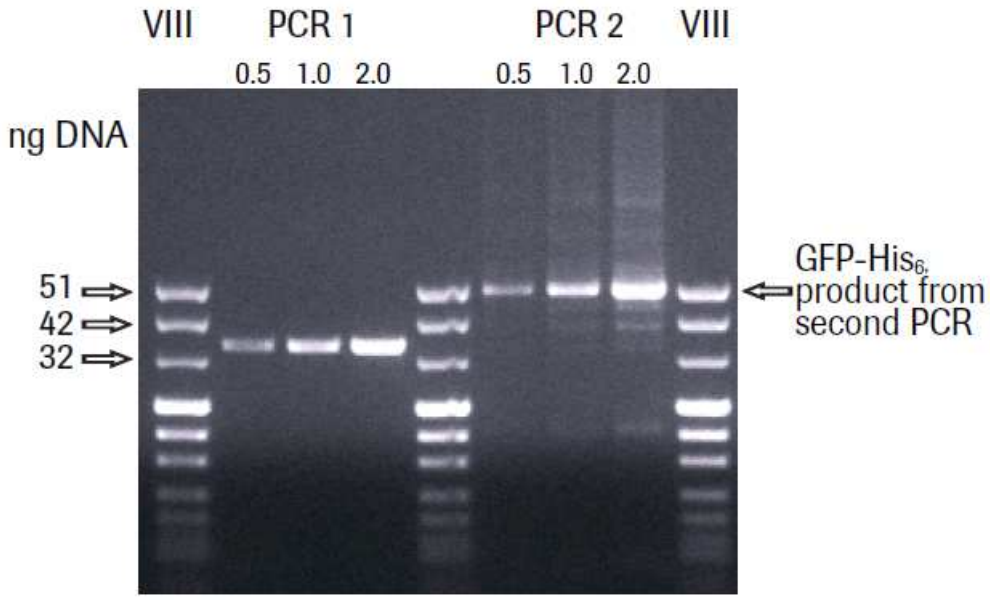

Figure 3. Typical products of the first and second PCR.

The first and second PCRs were performed with a sequence coding for GFP. Both PCR products were separated on a 1% agarose gel stained with 1 µg/ml ethidium bromide. The introduction of regulatory elements and a tag sequence in the second PCR reaction added approximately 370 bp to the product of the first PCR. The product of the second PCR was quantified densitometrically for expression in RTS 100 *E. coli* HY Kit. 0.5 µg of DNA molecular weight marker containing a defined amount of DNA/band was loaded and compared to 0.5, 1.0, and 2.0 µl of the product of the second PCR. Typical results were 50–100 ng/ µl DNA in a 50 µl PCR reaction volume.

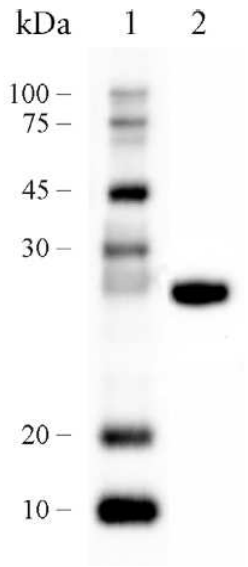

Figure 4. GFP expression from PCR template detected by Western blot analysis with the Anti-His_6 Peroxidase conjugate; 0.5 µl of the expression reaction was loaded on the gel.

PCR product from the second PCR coding for C-terminal His_6-tagged GFP was quantified (Figure 3). 150 ng of the PCR product (corresponding to 3 µl) were used for expression in a 50 µl reaction volume of the RTS 100 *E. coli* HY Kit and analyzed by SDS-PAGE and Western blotting (Figure 4). Best expression results were obtained with PCR product concentrations of 1–3 nM (corresponding to 50–150 ng of a 1 kb fragment in a 50 µl reaction volume).

Protocol 4: Generation of a linear control template

A linear control template can be generated by using the Control Vector GFP (green fluorescent protein) that is provided in the RTS 100 *E. coli* HY Kits.

Before starting

- GFP-specific primers that contain an overlap for introduction of a C-terminal His_6-tag must be ordered separately
- Order a set of desalted primers in a 0.02–0.04 µmol scale with the following sequences:

  C-terminal His_6-tag

  GFP-specific sense primer

  5'-CTTTAAGAAGGAGATATACC ATGACTAAAGGTGAAGAAC

  GFP-specific antisense primer

  5'- TGATGATGAGAACCCCCCCC TTGGTACAGTTCATCCAT

Procedure

1. Set up the first GFP-specific PCR according to Table 6.

   Use the Control Vector GFP from the RTS 100 *E. coli* HY Kit (bottle 6) as the template for the PCR.

Table 6. First GFP-specific PCR components

Component	Volume	Final concentration
Expand High Fidelity buffer, 10× conc. without $MgCl_2$	5 µl	1x
$MgCl_2$, stock solution (25 mM)	6 µl	3 mM
PCR Nucleotide Mix	1.25 µl	250 µM
GFP-specific sense primer	x µl	200 nM
GFP-specific antisense primer	y µl	200 nM
Expand High Fidelity Enzyme mix	0.85 µl	3 U
Template: Control Vector GFP (diluted 1:100)	1 µl	10 ng
PCR grade H_2O		Up to 50 µl

2. Set up the cycle profile for the thermocycler according to Table 7.

   Note: The cycle profile given is for the ABI GeneAmp 9600 Thermocycler. When using other thermocyclers, the cycle conditions must be adjusted.

Table 7. First PCR cycle profile

Cycles	Time, min	Temperature, °C	Remarks
1	4	94	Denaturation
20	1	94	Denaturation
	1	45	Annealing
	1	72	Elongation
1	4	72	Elongation

3. For the second PCR and addition of regulatory elements and HisTag to GFP, first purify the product of the first PCR with the GenUP™ PCR Cleanup Kit (cat. no. BR0700301, biotechrabbit).
4. Perform the second PCR according to the standard second PCR protocol in protocol 2, as described on page 11.
5. Analyze the products of the GFP-specific first and second PCR on a 1% agarose gel.

   The expected products are 754 and 1,121 bp, respectively (Figure 3).
6. Use 100–150 ng of the product of the second PCR for expression in a RTS 100 *E. coli* HY reaction and detect the synthesized GFP by Western blotting using the Anti-His_{6}-Peroxidase conjugate.

Supporting information

Cloning the generated PCR fragments

The Expand High Fidelity PCR System generates a mixture of PCR products with blunt ends and 3'-single A overhangs. Blunt end cloning and T/A cloning can be applied. Alternatively, PCR cloning techniques (e.g., In-Fusion™ technology offered by BD Bioscience Clontech, Palo Alto, CA) can be used at this step. After cloning, plasmid can be prepared in a large scale and used for expression in RTS 100, RTS 500, RTS 9000 *E. coli* HY Kits, and RTS Disulfide Kits. The sequence of the cloned PCR fragment should be verified before starting large scale plasmid preparation. If you want to clone the second PCR product using blunt end or T/A cloning and a T7 promoter, the orientation of the insert has to be checked. To avoid the generation of antisense mRNA, the selection of a clone with T7 promoters in the same orientation is recommended.

References

1. McPherson, M.J., Moller, S.G. 2000. “PCR” Bios Scientific Publishers Ltd, UK.
2. Newton, C.R., Graham, A. 1997. “PCR” Bios Scientific Publishers Ltd, UK.

Troubleshooting guide

The following troubleshooting recommendations are designed to address unexpected or undesired results. To ensure optimal use, follow the guidelines and recommendations in the manual.

First PCR

Observation	No product in first PCR
Possible cause	Template concentration too low
Resolving	Increase concentration of template.
Possible cause	Primer concentration or sequence not optimal
Resolving	Use high-quality primers.
Resolving	Verify primer sequences.
Resolving	Optimize primer concentration. Check primer concentrations on agarose gels and avoid imbalanced concentrations.
Resolving	Check the melting temperature, purity, and GC content of specific primers.
Possible cause	Extension time too short
Resolving	Increase extension time to 2 min/kb of PCR target.
Possible cause	Annealing temperature too high
Resolving	Lower the annealing temperature in 5°C increments.
Possible cause	Insufficient Mg^{2+} concentrations
Resolving	Optimize the Mg^{2+} concentration in a range of 1.0–4.5 mM.
Possible cause	Multiple contributing factors
Resolving	Test reaction with positive-control template and primers of known performance before starting experimental conditions.
Resolving	Start over, using fresh solutions of dNTPs, primers, and template.

Possible cause	Poor-quality template (degraded, contaminated, containing inhibitors)
Avoiding	Store template at –15 to –25°C in aliquots to avoid repeated freeze–thaw cycles.
Resolving	Prepare new template dilution.

Observation	Product band of first PCR not focused
Possible cause	Secondary amplification product
Avoiding	Check Mg^{2+} concentration and cycle conditions.
Avoiding	Optimize primer concentration.
Resolving	Raise annealing temperature in 3°C increments.
Resolving	Decrease the number of cycles.
Resolving	Decrease template concentration.

Observation	Nonspecific product bands in first PCR
Possible cause	Nonspecific binding of primers
Avoiding	Use higher annealing temperature to obtain the highest specificity.
Resolving	Check and optimize primer concentration.
Resolving	Redesign primers for more specific binding of target and/or to allow a higher annealing temperature.

Second PCR

Observation	No product in second PCR
Possible cause	Template concentration too low
Resolving	Check the concentration of the product of the first PCR on an agarose gel and increase the amount of template in the second PCR.

Possible cause	Extension time too short
Resolving	Increase the extension time to 2 min/kb of PCR target.

Possible cause	Insufficient Mg^{2+} concentrations
Resolving	Optimize the Mg^{2+} concentration in a range of 1.0–4.5 mM.

Possible cause	Multiple contributing factors
Avoiding	Test reaction with positive-control template and primers of known performance before starting experimental conditions.
Resolving	Start over, using fresh solutions of dNTPs, primers, and template.

Observation	Product band of second PCR not focused
Possible cause	Secondary amplification product
Avoiding	Check Mg^{2+} concentration and cycle conditions.
Resolving	Decrease the number of cycles.
Resolving	Decrease template concentration.

Observation	Nonspecific product bands in second PCR
Possible cause	Nonspecific binding of primers
Avoiding	Use higher annealing temperature to obtain the highest specificity.
Resolving	Check using an agarose gel if primers from the first PCR are completely used up. Decrease the amount of primers in the first PCR to 0.1 µM or purify the product from the first PCR from the agarose gel.
Resolving	Check if the T7 promoter and T7 terminator primers in the second PCR bind to the template DNA from the first PCR.

Possible cause	400 bp (approximate) byproduct
Resolving	Depending on the sequence of the gene-specific primers, a nonspecific by-product can appear in the second PCR caused by primer-dimer formation and carry-over contamination from the first PCR. In this case, reduce the primer and the $MgCl_2$ concentrations in the first PCR or purify the product of the first PCR from primer-dimers using the GenUP™ PCR Cleanup Kit or the GenUP™ Gel Extraction Kit (biotechrabbit).

Ordering information

Product	Size	Order no.
RTS Linear Template Kit Plus	20 reactions	BR1402401
RTS pIX3.0 Vector	1 vector, 25 µg	BR1402701
RTS 100 *E. coli* HY Kit	24 reactions	BR1400101
RTS 100 *E. coli* HY Kit	96 reactions	BR1400102
RTS 500 ProteoMaster E. coli HY Kit	5 reactions	BR1400201
RTS 9000 *E. coli* HY Kit	1 reaction	BR1400301
RTS 100 *E. coli* Disulfide Kit	24 reactions	BR1400401
RTS 500 *E. coli* Disulfide Kit	5 reactions	BR1400501
RTS 100 *E. coli* Fab Kit	10 reactions	BR1400601
RTS 100 *E. coli* Fab Kit	96 reactions	BR1400602
RTS pIVEX E. coli His-tag, 2nd Gen. Vector Set	2 vectors, 10 µg each	BR1400701
RTS Wheat Germ LinTempGenSet, His6-tag	96 reactions	BR1401201
RTS pIVEX Wheat Germ His6-tag Vector Set	2 vectors, 10 µg each	BR1401301
RTS 100 Wheat Germ CECF Kit	24 reactions	BR1401001
RTS 500 Wheat Germ CECF Kit	5 reactions	BR1401101
RTS 500 Adapter	1 adapter	BR1401901
RTS GroE Supplement	For five RTS 500 reactions	BR1401701
RTS DnaK Supplement	For five RTS 500 reactions	BR1401601
RTS Amino Acid Sampler	1 set	BR1401801
RTS 100 Insect Membrane Kit	5 reactions	BR1401501
RTS 100 Insect Membrane Kit	20 reactions	BR1401502
RTS 100 Insect Disulfide Kit	10 reactions	BR1401401
RTS 100 Insect Disulfide Kit	96 reactions	BR1401402
RTS Linear Template Fab Kit	96 reactions	BR1402201
RTS pIX4.0 Insect Vector	1 vector, 25 µg	BR1400901