RTS™ Wheat Germ LinTempGenSet, His_6-tag Manual

For rapid production of linear expression templates using PCR

RTS Wheat Germ LinTempGenSet, His_6-tag, April, 2015

Contents

Product specifications

The RTS Wheat Germ LinTempGenSet, His_6-tag is a module to rapidly generate linear expression constructs by PCR. The constructs are ready for use as DNA templates in RTS Wheat Germ Continuous Exchange Cell-Free (CECF) *in vitro* protein expression reactions without the need for purification.

Product description

The RTS Wheat Germ LinTempGenSet, His_6-tag provides the components and procedures necessary for generation of linear expression constructs by PCR (96 reactions).

Product limitations

The RTS Wheat Germ LinTempGenSet, His_6-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

RTS Wheat Germ LinTempGenSet, His_6-tag

Vial	Content and function	No. per kit
	Ordering number	BR1401201
1	Forward Primer; WG LTGS	4 vials (24 reactions each)
2	Reverse Primer; WG LTGS	4 vials (24 reactions each)
3	C-terminal His_6-taq DNA; WG LTGS (coding for a C-terminal His_6-tag and all regulatory elements necessary for *in vitro* protein expression)	1 vial (96 reactions)
4	N-terminal His_6-tag DNA; WG LTGS (coding for a N-terminal His_6-tag and all regulatory elements necessary for *in vitro* protein expression)	1 vial (96 reactions)
5	RNase-DNase-free Water; WG 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:

- Thermostable DNA polymerase (e.g. Expand™ High Fidelity PCR System (Roche) or Hot Start Taq DNA Polymerase (cat. no. BR0200101, biotechrabbit)
- PCR Nucleotide Mix
- DNA Molecular Weight Marker
- Agarose
- 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 Wheat Germ LinTempGenSet, His_6-tag is shipped on dry ice.

RTS Wheat Germ LinTempGenSet, His_6-tag should be stored dry in the dark 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. Certificate of analysis sheets for biotechrabbit products can be obtained 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 Wheat Germ product line combines a series of technologies for efficient and optimized protein expression in eukaryotic cell lysates. It overcomes the limitations that often restrict the use of *E. coli*-based systems (cell-based or cell-free), especially with eukaryotic target proteins. A number of innovative methods have been incorporated in this system, including expression from PCR-generated linear templates, rapid generation of expression plasmids without restriction cloning, fast screening for optimized reaction time and temperature, high yield *in vitro* expression using the CECF principle, and optimized lysate biochemistry. In most cases, eukaryotic proteins are produced with much higher success rates and better solubility in RTS Wheat Germ than in bacterial lysates, with sequence optimization being generally not required.

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 promoter, eukaryotic 5' and 3' 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 5' and 3' ends are extended. This linear expression construct is finally amplified via the flanking primers (Figure 1).

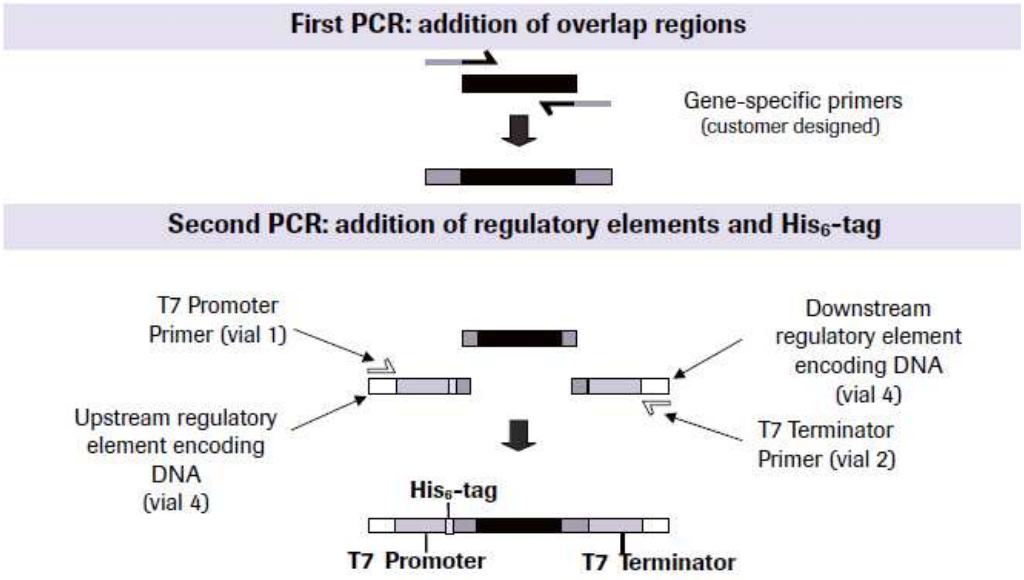

Figure 1. Principle of the generation of PCR products for *in vitro* protein expression via the RTS Wheat Germ LinTempGenSet, His_6-tag. As an example, the introduction of an N-terminal His_6-tag is shown. The two-step PCR will result in the addition of nucleotides to both ends of the original cDNA.

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. The use of PCR-generated templates is one of the major advantages of cell-free expression systems. If primers are designed for the first (gene specific) of these two PCRs according to the instructions provided in this manual, the resulting product can be used in a second PCR to produce a completely functional linear expression template for expression in either RTS 100 *E. coli* HY (using the primers provided in the RTS Linear Template Kit Plus) or RTS 100 Wheat Germ CECF Kit (using the primers provided in this kit).

At a later step in the RTS workflow, cloning into an RTS pIVEX vector will usually be required (e.g. to produce a template for expression scale-up, or to make sure a well characterized and stabilized template is used in a series of subsequent experiments). Standard cloning via restriction sites or PCR Cloning techniques (e.g. In-Fusion™ technology) are a time-saving and efficient alternative to traditional cloning at this step.

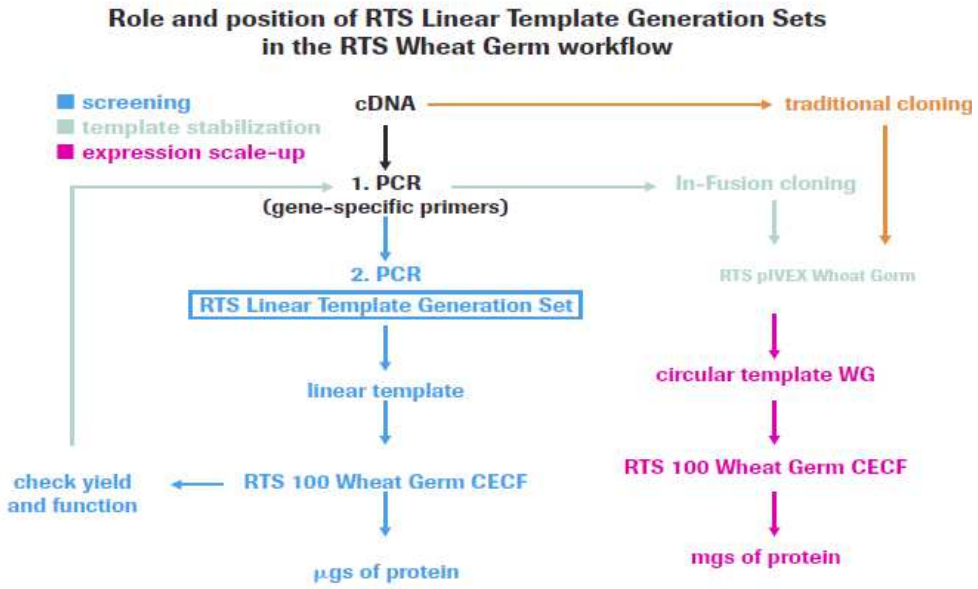

Figure 2. Integration of the RTS LinTempGenSet (boxed) into the RTS workflow.

Advantages of the RTS Wheat Germ LinTempGenSet, His_6-tag include:

- Rapid creation of linear expression constructs
- Optimized regulatory elements
- No laborious cloning
- Flexible positioning of His_6-tag

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

Before starting

- All nucleotide sequences can be downloaded from www.biotechrabbit.com
- biotechrabbit recommends standard-quality (HPLC purified) 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 (please note that the ATG is essential)	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 (please note that the ATG is essential)	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 other than RTS pIVEX WG (e.g. pIVEX *E. coli*, 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

Please note, that the protocol is an example for the **Expand™ High Fidelity PCR System**. When using another enzyme, please follow the instructions of the corresponding DNA polymerase.

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 µl	1.5 mM
PCR Nucleotide Mix	1.25 µl	250 µM
Gene-specific sense primer	x µl	100–300 nM
Gene-specific antisense primer	y µl	100–300 nM
Expand High Fidelity Enzyme mix	0.8 µl	2.8 U
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
20–25	1 min	94	Denaturation
	1 min	45–60*	Annealing
	45 s†	72	Elongation
1	Up to 10 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 Table 4.

Table 4. Preparation of working solutions

Component	Preparation	Final concentration
Forward Primer; WG LTGS (vial 1)	Add 80 µl H_2O, PCR-grade (vial 5)	6 µM
Reverse Primer; WG LTGS (vial 2)	Add 80 µl H_2O, PCR-grade (vial 5)	6 µM
C-terminal His_6-tag DNA; WG LTGS (vial 3)	Add 90 µl H_2O, PCR-grade (vial 5) 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)	50x
N-terminal His_6-tag DNA; WG LTGS (vial 4)	Add 90 µl H_2O, PCR-grade (vial 5) 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)	50 x

2. 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).

3. 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 using the RTS 100 Wheat Germ CECF 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.

4. Set up the second PCR according to Table 5.

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

Table 5. 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)	3 µl	1.5 mM
PCR Nucleotide Mix	1.25 µl	250 µM each
Vial 1 working solution: Forward Primer (6 µM)	4 µl	480 nM
Vial 2 working solution: Reverse 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	0.8 µl	2.8 U
Template: Product of the first PCR	2–4 µl	150–300 ng
PCR grade H_2O		Up to 50 µl

5. Set up the cycle profile for the thermocycler according to Table 6.

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

Table 6. Second PCR cycle profile

Cycles	Time	Temperature, °C	Remarks
1	4 min	94	Denaturation
20–25	1 min	94	Denaturation
	1 min	50	Annealing
	1 min*	72	Elongation
1	Up to 10 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 Wheat Germ CECF 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 1–1.5% 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 1 µg product of the second PCR for expression in a 50 µl reaction volume with RTS 100 Wheat Germ CECF Kit (Figure 4).

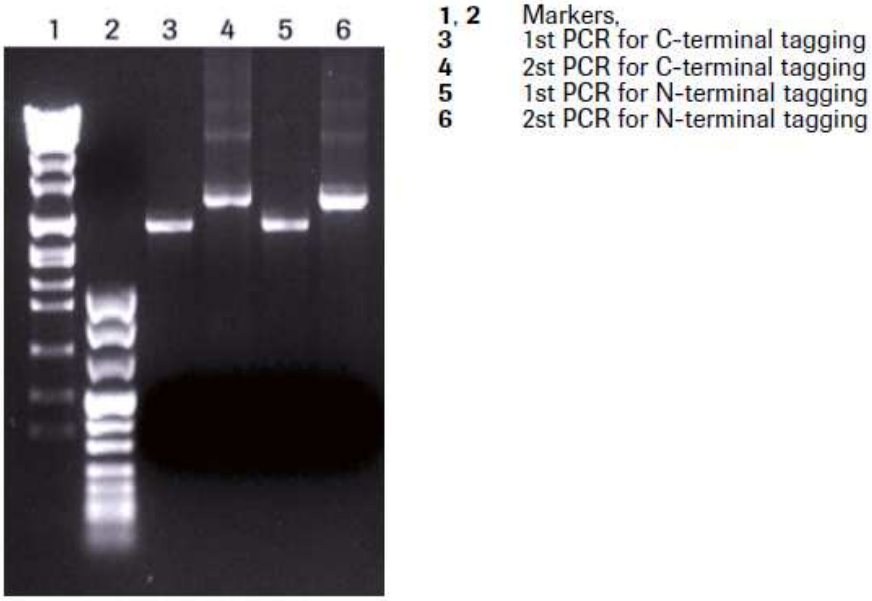

Figure 3. Typical products of the first and second PCR for C- and N-terminally tagged linear templates coding for GUS.

The first and second PCRs were performed with a sequence coding for glucuronidase (GUS). 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 473 bp to the product of the first PCR. The product of the second PCR was quantified densitometrically for expression in RTS 100 Wheat Germ CECF Kit. 0.5 µg of DNA molecular weight marker (VII and VIII, Roche) 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 70–100 ng/ µl DNA in a 50 µl PCR reaction volume.

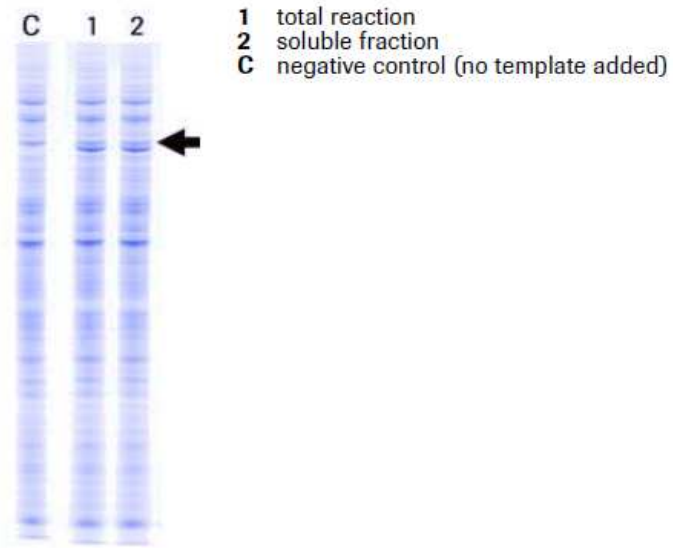

Figure 4. Expression of a linear template (product of second PCR) encoding an N-terminal His_6-tag. 0.5 µl of the expression reactions were loaded on the gel after a 24-hour reaction at 24°C.

PCR product from the second PCR coding for N-terminal His_6-tagged GUS was quantified (Figure 3). 1 µg of the PCR product (corresponding to 10 µl) was used for expression in a 50 µl reaction volume of the RTS 100 Wheat Germ CECF Kit and analyzed by SDS-PAGE (Figure 4) and optionally by Western blotting.

Protocol 4: Generation of a linear control template

A linear control template can be generated by using the GUS Control Vector provided in the RTS 100 Wheat Germ CECF Kit.

Procedure

1. Prepare the working solutions according to Table 7.

Table 7. Preparation of working solutions

Component	Preparation	Final concentration
Forward Primer; WG LTGS (vial 1)	Add 80 µl H_2O, PCR-grade (vial 5)	6 µM
Reverse Primer; WG LTGS (vial 2)	Add 80 µl H_2O, PCR-grade (vial 5)	6 µM

2. Set up the GUS-specific PCR according to Table 8.

   Use the GUS Control Vector from the RTS 100 Wheat Germ CECF Kit as the template for the PCR.

Table 8. 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 µl	1.5 mM
PCR Nucleotide Mix	1.25 µl	250 µM
Forward primer; 6 µM (Vial 1 working solution)	4 µl	480 nM
Reverse primer; 6 µM (Vial 2 working solution)	4 µl	480 nM
Expand High Fidelity Enzyme mix	0.8 µl	2.8 U
Template: GUS Control Vector (diluted 1:10)	2 µl	200 ng
PCR grade H_2O	30 µl	

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

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

Table 9. PCR cycle profile

Cycles	Time, min	Temperature, °C	Remarks
1	4	94	Denaturation
20	1	94	Denaturation
	1	50	Annealing
	2.4	72	Elongation
1	Up to 10	72	Elongation

4. Analyze the products of the GUS-specific PCR on a 1% agarose gel.
   The expected product is 2,300 bp.

Supporting information

Cloning the PCR fragments generated

The Expand High Fidelity PCR System generates a mixture of PCR products with blunt ends and 3'-single A overhangs. Subsequently in the RTS workflow, insertion of these products into an RTS pIVEX Wheat Germ vector will generally be required (e.g. to obtain a template suitable for expression scale-up to RTS 500 Wheat Germ CECF). Standard cloning via restriction sites or 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, plasmids can be prepared on a large scale using GenUP™ Plasmid Kit or GenUP™ Plasmid Plus Kit (cat. no. BR0700201, BR0701201, biotechrabbit). The sequence of the cloned PCR fragment should be verified before starting large-scale plasmid preparation.

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 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 forward and reverse 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 byproduct 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 (biotechrabbit) 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