

Replicator™ RNAi Kit

Product code: F-610

Stable for one year from the packaging date.
Store at -20°C.

1. Description

Replicator™ RNAi Kit* is a highly efficient and specific tool for generating double-stranded RNA for gene silencing experiments. The kit utilizes a new technology where dsRNA is produced by combined *in vitro* transcription and replication reactions. Both reactions take place in a single incubation step.

The DNA template for the dsRNA synthesis is produced by PCR using Phusion™ High-Fidelity DNA Polymerase. Phusion DNA Polymerase's high speed, fidelity and robustness ensure correct starting material for the dsRNA production and enable amplification of long templates up to 10 kb.

The PCR product is transcribed into single-stranded RNA by T7 RNA Polymerase. The ssRNA is then replicated into double-stranded RNA by Phi6 RNA Replicase¹⁻³ in the same incubation step. Due to the unique combination of complementary polymerase activities, the produced dsRNA is perfectly duplexed.

The reaction yield is high. The kit contains sufficient reagents for producing up to 2.5 mg of dsRNA in a total volume of 2 ml. The yield depends on the target sequence.

* Patent pending

2. Applications

dsRNA produced with this kit is a suitable substrate for enzymes such as RNase III and Dicer, which cut the dsRNA into small interfering RNAs and thereby produce a pool of siRNA targeted for a chosen sequence. Such siRNA pools have successfully been used in gene silencing experiments⁴⁻⁶.

3. Kit Components

Component	Volume
Phusion™ High-Fidelity DNA Polymerase (2 U/μl)	25 μl
10 mM dNTP mix	20 μl
5x Phusion HF buffer	1.5 ml
Lambda control DNA (0.5 ng/μl)	20 μl
Control Primer mix (25 μM each)	20 μl
Phi6 RNA Replicase	60 μl
T7 RNA Polymerase (New England Biolabs)	60 μl
Pyrophosphatase	40 μl
10x dsRNA Synthesis Buffer	250 μl
5x NTP mix	400 μl
50 mM MnCl ₂	500 μl
8 M LiCl	2 ml

4. Shipping and Storage

The Replicator RNAi Kit is shipped in gel ice. Upon arrival, store the kit components at -20°C. The 10x dsRNA Synthesis Buffer contains DTT which may lose its reducing capability when stored or handled inappropriately. LiCl solution can also be stored at room temperature. The components are stable for one year from the date of packaging when stored properly.

5. Method Overview

The dsRNA is synthesized by combined *in vitro* transcription and replication from a DNA template.

The DNA template for the dsRNA synthesis is produced by PCR using Phusion High-Fidelity DNA Polymerase. The PCR primers are designed so that the resulting PCR product contains the target sequence flanked by T7 promoter sequence at one end and Phi6 RNA Replicase promoter sequence at the other end.

The PCR product is purified and transcribed into single-stranded RNA by T7 RNA Polymerase. This ssRNA is replicated into double-stranded RNA by Phi6 RNA Replicase in the same incubation step.

6. Template Preparation by PCR

6.1 Target preparation

With the Replicator RNAi Kit, targets ranging between 100 bp and several kilobases in length can be amplified. The template DNA for the PCR can be either genomic DNA, plasmid DNA or cDNA produced using reverse transcriptase enzyme.

6.2 Primer design

The primers have to be designed separately for each target sequence. RNA polymerase promoter sequences needed for the dsRNA synthesis are added to both sides of the target sequence using PCR. The PCR primers are designed so that they contain RNA polymerase promoter sequences at their 5' ends. Thus in the PCR product, RNA polymerase promoter sequences flank the target sequence. In addition to promoter sequences, each primer should contain 17-22 nucleotides of target gene-specific sequence at the 3' end.

The PCR amplification primers are illustrated in figure 1. The promoter sequences are underlined. The first nucleotides to be incorporated into the final dsRNA product at each end are marked in red. Target gene-specific sequences at the 3' end of the primers are denoted by N₍₁₇₋₂₂₎.

Figure 1. Amplification primers.

Forward primer	5' <u>TAATACGACTCACTATA</u> <u>GGN</u> ₍₁₇₋₂₂₎ 3'
Reverse primer	5' <u>GGAAAAAAN</u> ₍₁₇₋₂₂₎ 3'

6.3 PCR

PCR reactions should be set up on ice. It is critical that the Phusion DNA Polymerase is the last component added to the PCR mixture, since the enzyme exhibits 3'-5' exonuclease activity that can degrade primers in the absence of dNTPs. Phusion DNA Polymerase produces blunt end DNA products.

The optimal amount of enzyme depends on the length of the PCR product and the amount of template used. Normally, 1 unit of Phusion DNA Polymerase per 50 μl reaction volume gives good results. Optimal enzyme amount can range from 0.5-2 units per 50 μl reaction depending on the amplicon length and difficulty. Do not exceed 2 units per 50 μl reaction. For optimal results, use 200 μM of high quality dNTPs included in the kit.

The amount of template depends on the complexity of DNA. Use 1 pg to 10 ng template per 50 μl reaction with low complexity DNA (e.g. plasmid, lambda or BAC DNA) or 50 to 200 ng template per 50 μl reaction with high complexity genomic DNA. If the amplification is done directly from cDNA, use no more than five microliters of unpurified reverse transcription reaction in a 50 μl reaction.

PCR and dsRNA production efficiency can be controlled using the accompanied λ control DNA and control primers (see Tables 1B and 2B).

Table 1A. Pipetting instructions for the PCR reaction.

Component (in order)	Volume / 50 μl reaction	Final concentration
H ₂ O	add to 50 μl	
5x Phusion HF buffer	10 μl	1x
dNTPs (10 mM)	1 μl	200 μM
Forward primer	x μl	0.5 μM
Reverse primer	x μl	0.5 μM
Template DNA	x μl	
Phusion DNA Polymerase	0.5 μl	0.02 U/μl

Table 1B. Pipetting instructions for the control PCR reaction.

Component	Volume / 50 µl reaction
H ₂ O	33.5 µl
5x Phusion HF buffer	10 µl
dNTPs (10 mM)	1 µl
Control primer mix (25 µM each)	1 µl
λ control DNA (0.5 ng/µl)	4 µl
Phusion DNA Polymerase	0.5 µl

6.3.1 Cycling conditions

Due to the novel nature of Phusion DNA Polymerase, optimal reaction conditions may differ from standard enzyme protocols. Phusion DNA Polymerase tends to work better at elevated denaturation and annealing temperatures due to higher salt concentrations in its buffer.

Table 2A. Cycling instructions.

Cycle step	Temperature	Time	Cycle number
Initial denaturation	98°C	30 s	1
Denaturation	98°C	5-10 s	25-35
Annealing	45-72°C	10-30 s	
Extension	72°C	15-30 s / 1 kb	
Final extension	72°C 4°C	5-10 min hold	1

Table 2B. Cycling instructions for the control reaction.

Cycle step	Temperature	Time	Cycle number
Initial denaturation	98°C	30 s	1
Denaturation	98°C	10 s	34
Annealing, extension	72°C	35 s	
Final extension	72°C 4°C	5 min hold	1

Initial denaturation

30 s initial denaturation at 98°C is recommended for most templates. Some templates may require longer initial denaturation and the length of the initial denaturation time can be extended up to 3 minutes.

Denaturation

Keep the denaturation as short as possible. Usually 5-10 seconds at 98°C is enough for most templates.

Primer annealing

The Phusion DNA Polymerase has the ability to stabilize primer-template hybridization. As a basic rule, for primers >20 nt, anneal for 10-30 seconds at a T_m +3°C of the lower T_m primer. For best results, calculate the T_m for the target gene-specific sequence of the primers. The T_m's should be calculated with the nearest-neighbor method, because results from primer T_m calculations can vary significantly depending on the method used. Instructions for T_m calculation and a link to a calculator using the nearest-neighbor method can be found on Finnzymes' website (www.finnzymes.com). For primers ≤20 nt, use an annealing temperature equal to the T_m of the lower T_m primer. In case the annealing temperature approaches 72°C, a two-step cycling protocol without a separate annealing step is recommended.

Extension

The extension should be performed at 72°C. Extension time depends on amplicon length and complexity. For low complexity DNA (e.g. plasmid, lambda or BAC DNA) use extension time 15 s per 1 kb. For high complexity genomic DNA 30 s per 1 kb is recommended.

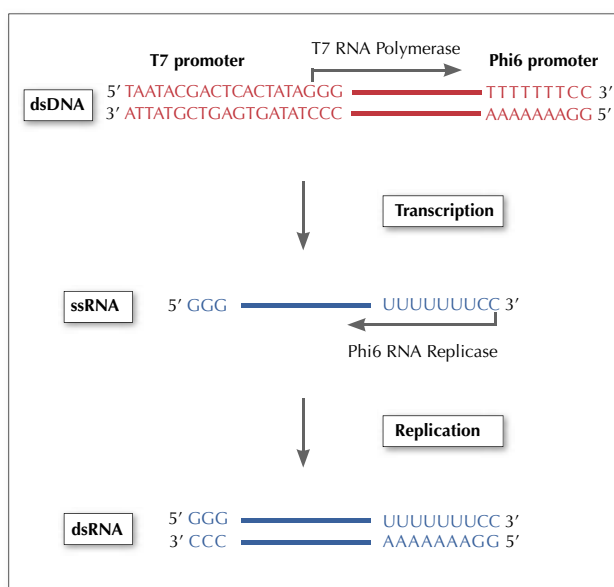
More information on using Phusion DNA Polymerase can be found at Finnzymes' web page: www.finnzymes.com

6.4 Purification

Following PCR, the size and purity of the PCR product should be examined on an agarose gel. The PCR product can be purified using either phenol/chloroform extraction followed by standard ethanol precipitation or commercially available DNA purification columns. Alternatively, the PCR product can be gel-purified if necessary. Resuspend the DNA in RNase-free water or 10 mM Tris-HCl, pH 8.0-8.5. The concentration of the PCR product can be determined by spectrophotometric measurement at 260 nm or estimated from an agarose gel. The expected length of the PCR product from the control reaction is 881 bp.

7. dsRNA Production Setup

dsRNA is produced in a single reaction combining DNA transcription and replication of the ssRNA into dsRNA. The dsRNA synthesis reaction is illustrated in figure 2.

Figure 2. dsRNA synthesis reaction.

The reaction volume can be scaled up from the 50 µl described in Table 3. It is advisable to perform a single 20–50 µl pilot reaction before scale up to confirm all reaction conditions are correct. Use of RNase inhibitor is not normally necessary because ssRNA produced by T7 RNA Polymerase is immediately replicated into more stable dsRNA by Phi6 RNA Replicase.

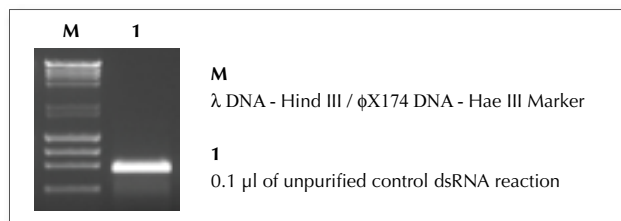
1. Thaw the reagents (10x dsRNA Synthesis Buffer, 5x NTP mix and MnCl₂) at room temperature immediately before use. Keep the 10x dsRNA Synthesis Buffer and MnCl₂ at room temperature until use. Store the ribonucleotides and enzyme solutions on ice until use.
2. Prepare the dsRNA synthesis reaction by combining the reaction components in the following order. Reaction assembly should be done at room temperature because the dsRNA synthesis buffer contains spermidine that can precipitate nucleic acids on ice. Use 0.5–2 µg of DNA template per 50 µl reaction. Set up a separate reaction for each template of interest. It is advisable to perform a concomitant control reaction using the control PCR product to test the reaction conditions.

Table 3. Pipetting instructions for the dsRNA reaction.

Components (in order)	Volume / 50 µl reaction	Final concentration
RNase-free water	add to 50 µl	
10x dsRNA Synthesis Buffer	5 µl	1x
5x NTP mix	10 µl	1x
DNA template	x µl	10–40 ng/µl
MnCl ₂ (50 mM)	1.5 µl	1.5 mM
Pyrophosphatase	1 µl	
T7 RNA Polymerase	1.5 µl	
Phi6 RNA Replicase	1.5 µl	

- Mix the components thoroughly. Spin briefly to collect the reaction mixture at the bottom of the tube.
- Incubate at 35°C for 2–4 hours using e.g. a thermal cycler. The reaction can also be allowed to proceed overnight. Overnight incubation doubles the yield of some dsRNAs.
- It is recommended to examine the dsRNA product by standard agarose gel electrophoresis. Take a 5 µl sample from the dsRNA reaction and dilute it 20- to 100-fold before the gel electrophoresis. The gel and electrophoresis buffer should be prepared with RNase-free water. Common DNA markers can be used to estimate the length of the dsRNA product with sufficient accuracy. The control reaction should yield a dsRNA of 864 bp (figure 3). Occasionally, some ssRNA is visible after the reaction. ssRNA migrates on the gel at approximately half the size of the corresponding dsRNA.

Figure 3. Control dsRNA reaction.



The yield of dsRNA from a 50 µl reaction is typically 20–60 µg, depending on the target sequence and length.

The dsRNA synthesis reaction can be stored at -20°C or -80°C before purification. Storage of unpurified dsRNA may cause the formation of a white precipitate. The precipitate does not contain RNA and can be removed by centrifugation before purification.

8. Purification of dsRNA

The need for purification depends on the downstream application. Double-stranded RNA can be purified from contaminating reaction components using either selective LiCl precipitation or nuclease digestion. LiCl solution is included in the kit. Both purification methods may require an additional NTP removal step (e.g. gel filtration), depending on the use of the dsRNA product.

LiCl precipitation

LiCl precipitation selectively removes template DNA and ssRNA from the reaction. The dsRNA synthesis reaction is first precipitated with 2 M LiCl which precipitates all or most of the ssRNA present. The supernatant containing the dsRNA is then exposed to a second precipitation step using 4 M LiCl which efficiently precipitates dsRNA leaving template DNA and most NTPs in the supernatant. Keep the reactions on ice while pipetting.

- Add 1/3 volume of 8 M LiCl to the dsRNA synthesis reaction in a 1.5 ml microcentrifuge tube and mix. The final LiCl concentration is 2 M.
- Incubate at -20°C for 30 minutes.
- Centrifuge at 14 000 rpm in a microcentrifuge for 20 minutes at 4°C.
- Carefully separate the supernatant from the pellet. The dsRNA remains in the supernatant, while ssRNA is precipitated.
- Add 1/2 volume of 8 M LiCl to the supernatant. The final LiCl concentration is 4 M. This step precipitates dsRNA.
- Incubate at -20°C for 30 minutes.
- Centrifuge at 14 000 rpm for 20 minutes at 4°C.
- Remove the supernatant. The dsRNA pellet is not necessarily visible. Wash the pellet with 500 µl of 70% (v/v) ethanol.
- Centrifuge at 14 000 rpm for 5 minutes at 4°C.
- Carefully remove the ethanol without disturbing the pellet.
- Air-dry the pellet for 5-10 minutes at room temperature and resuspend the dsRNA in 50 µl of RNase-free water or TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Be careful not to over-dry the pellet, as it may become difficult to resuspend the dsRNA.

Nuclease treatment

Alternatively, the template DNA and ssRNA can be removed from the synthesis reaction by DNase (RNase-free) and RNase digestion. Perform the reactions according to the manufacturer's instructions. Make sure to choose nucleases that do not degrade double-stranded RNA.

Gel filtration

After LiCl precipitation or nuclease treatment, the dsRNA may be further purified using commercial gel filtration columns to ensure that all NTPs are removed from the reaction. Removing remaining NTPs will allow accurate spectrophotometric quantitation of dsRNA.

Analysis by gel electrophoresis

It is recommended to analyze the purified dsRNA using agarose gel electrophoresis. The size of dsRNA can be estimated comparing to common DNA markers.

9. Quantitation and Storage of the dsRNA

The concentration of dsRNA can be determined spectrophotometrically at 260 nm. In order to obtain accurate quantitation, the dsRNA has to be free from contaminating NTPs. Measure the absorbance at 260 nm from a 1:4 or 1:20 dilution. For accurate results, make the dilutions using 10 mM Tris-HCl, pH 7.5. The concentration of RNA can be calculated as follows: 1 $A_{260} = 40 \mu\text{g}$ dsRNA per ml. Store the purified RNA at -20°C or -80°C.

Purity of RNA

The ratio of absorbances at 260 and 280 nm (A_{260}/A_{280}) provides an estimate of the purity of RNA with respect to contaminants that absorb in the UV region, such as proteins. Pure RNA should exhibit an A_{260}/A_{280} ratio of 1.9-2.1 in 10 mM Tris-HCl, pH 7.5. The A_{260}/A_{280} ratio is influenced by pH and since water is not buffered, it is recommended to measure the absorbance of RNA sample in 10 mM Tris-HCl, pH 7.5.

10. Troubleshooting

PCR
No product at all or low yield
<ul style="list-style-type: none"> • Template concentration may be too low. Use more template. • Lengthen extension time. • Increase the number of cycles. • Lower annealing temperature. • Optimize enzyme concentration. • Denaturation temperature may be too low. Optimal denaturation temperature for most templates is 98°C or higher. • Denaturation time may be too long or too short. Optimize denaturation time. • Check purity and concentration of the primers. • Check primer design.
Non-specific products - High molecular weight smear
<ul style="list-style-type: none"> • Reduce enzyme concentration. • Shorten extension time. • Reduce total number of cycles. • Increase annealing temperature or try a two-step protocol (see 6.3.1). • Perform PCR setup on ice. Transfer the tubes directly to pre-heated PCR block.
Non-specific products - Low molecular weight discrete bands
<ul style="list-style-type: none"> • Raise annealing temperature. • Shorten extension time. • Lower enzyme concentration. • Titrate template amount. • Lower primer concentration. • Design new primers. • Perform PCR setup on ice. Transfer the tubes directly to pre-heated PCR block.

More troubleshooting for PCR can be found at www.finnzymes.com

dsRNA synthesis reaction	
Low yield	
Possible Causes	Comments and Suggestions
Spermidine precipitates DNA template	Assemble the reaction at room temperature. Spermidine may precipitate if the reaction is assembled on ice.
Not enough active DTT in the buffer	The reducing capacity of DTT decreases gradually in the course of time. Add fresh DTT (10 mM).
RNA is degraded	RNase contamination present in the reaction or DNA template. Be careful to avoid RNase contamination. Optionally, RNase inhibitor can also be included in the reaction.
Incubation time too short	Increase the incubation time.
Difficult template	Some sequences are inherently difficult templates. Design new primers for the target template.
DNA template	Check the concentration of the DNA template. Inefficient purification may leave contaminating dNTPs which cause inaccurate spectrophotometric quantitation.
Multiple reaction products	
Possible Causes	Comments and Suggestions
ssRNA	ssRNA migrates at half the size of the corresponding dsRNA on an agarose gel. ssRNA production is sequence specific and may be reduced by designing new primers for the target template.
Non-specific dsRNA from non-specific PCR products	dsRNA is also synthesized from non-specific PCR products. Check that the PCR reaction does not contain multiple products. Refer to PCR troubleshooting section for PCR optimization. If necessary, purify the PCR product from an agarose gel.
Smear	
Possible Causes	Comments and Suggestions
The reaction products are degraded on the gel	Make sure that the gel is prepared using RNase-free water to avoid RNase contamination.
dsRNA degraded by nuclease treatment	If dsRNA is purified using nucleases, make sure to choose nucleases that do not degrade dsRNA.

LiCl precipitation	
Low yield	
Possible Causes	Comments and Suggestions
Inefficient precipitation of dsRNA at 4 M LiCl concentration	Incubate the precipitation reaction at -20°C before each centrifugation (excluding washing step). Skipping the incubation step may decrease the yield slightly, especially with short templates.
dsRNA pellet lost	Make sure not to disturb the pellet during pipetting to ensure the pellet is not lost.
dsRNA not purified	
Possible Causes	Comments and Suggestions
Inefficient precipitation of ssRNA at 2 M LiCl concentration	<ul style="list-style-type: none"> Incubate the precipitation reaction at -20°C before each centrifugation. Skipping the incubation step may cause insufficient precipitation of the ssRNA. At some instances, all ssRNA is not removed by the 2 M LiCl precipitation step. Use RNases to remove the ssRNA.

11. References

1. Makeyev E.V. and Bamford D.H. (2000) *EMBO J* 19: 124-133.
2. Makeyev E.V. and Bamford D.H. (2000) *EMBO J* 19: 6275-6284.
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4. Calegari F. et al. (2002) *Proc Natl Acad Sci U S A* 99: 14236-14240.
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6. Yang D. et al. (2002) *Proc Natl Acad Sci U S A* 99: 9942-9947.

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