

GeneSilencer[®] PCR Kits Instruction Manual

Catalog Numbers

P140100

P640100

P140300

P640300



Genlantis

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Limited License

The purchase price paid for the GeneSilencer[®] PCR Kits by end users grants them a non-transferable, non-exclusive license to use the kits and included components (as listed in the Contents section). These kits are intended for **internal research only** by the purchaser. Such use is limited to the generation of siRNAs and subsequent transfer of the siRNAs into cells or animals by licensed facilities. Furthermore, **internal research only** use means that the GeneSilencer PCR Kits and all of their contents are excluded, without limitation, from resale, repackaging, or use for the making or selling of any commercial product or service without the written approval of Genlantis, a division of Gene Therapy Systems, Inc ("GTS").

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The GeneSilencer[®] PCR Kits and all of their components are developed, designed, intended, and sold for research use only. They are not to be used for human diagnostic or included/used in any drug intended for human use. All care and attention should be exercised in the handling of the kit components by following appropriate research lab practices.

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Director of Licensing

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OVERVIEW

Available Kits and Contents

The following GeneSilencer® PCR kits are currently available from Genlantis.

Product Name	Description	Kit Contents	Catalog Number
GeneSilencer® H1 PCR Kit (20 reactions)	For transcribing siRNAs using the H1 RNA Pol III promoter	1 x 100 µl H1 Template, 10 ng/µl 1 x 20 µl H1 Upstream Primer, 1 µg/µl 1 x 20 µl H1 Downstream Control Sense Primer (Luc.), 1 µg/µl 1 x 20 µl H1 Downstream Control Antisense Primer (Luc.), 1 µg/µl	P140100
GeneSilencer® U6 PCR Kit (20 reactions)	For transcribing siRNAs using the U6 RNA Pol III promoter	1 x 100 µl U6 Template, 10 ng/µl 1 x 20 µl U6 Upstream Primer, 1 µg/µl 1 x 20 µl U6 Downstream Control Sense Primer (Luc.), 1 µg/µl 1 x 20 µl U6 Downstream Control Antisense Primer (Luc.), 1 µg/µl	P640100
GeneSilencer® H1-GFP PCR Kit (20 reactions)	For transcribing siRNAs fused to GFP using the H1 RNA Pol III promoter.	1 x 100 µl H1-GFP Template, 10 ng/µl 1 x 20 µl H1-GFP Upstream Primer, 1 µg/µl 1 x 20 µl H1-GFP Downstream Control Sense Primer (Luc.), 1 µg/µl 1 x 20 µl H1-GFP Downstream Control Antisense Primer (Luc.), 1 µg/µl	P140300
GeneSilencer® U6-GFP PCR Kit (20 reactions)	For transcribing siRNAs fused to GFP using the U6 RNA Pol III promoter.	1 x 100 µl U6-GFP Template, 10 ng/µl 1 x 20 µl U6-GFP Upstream Primer, 1 µg/µl 1 x 20 µl U6-GFP Downstream Control Sense Primer (Luc.), 1 µg/µl 1 x 20 µl U6-GFP Downstream Control Antisense Primer (Luc.), 1 µg/µl	P640300

Shipping and Storage

The GeneSilencer® PCR kits are shipped frozen. For maximum stability, we recommend that you store the siRNA Expression Vector Kits at –20°C upon receipt.

Accessory Products

GTS offers the following products for use in conjunction with the GeneSilencer® PCR kits.

For efficient transfection of siRNAs into cells

Product Name	Catalog Number	Quantity
GeneSilencer® siRNA Transfection Reagent	T500750	200 reactions (0.75 ml)
GeneSilencer® siRNA Transfection Reagent	T505750	5 x 200 reactions (5 x 0.75 ml)

For efficient transcription of shRNAs into cells

Product Name	Catalog Number	Quantity
GeneSilencer® pGSH1 shRNA Vector Kit	P100100	25 reactions
GeneSilencer® pGSU6 shRNA Vector Kit	P600100	25 reactions
GeneSilencer® pGSH1-GFP shRNA Vector Kit	P100300	25 reactions
GeneSilencer® pGSU6-GFP shRNA Vector Kit	P600300	25 reactions

For in vitro generation and transfection of heterologous pools of siRNAs

Product Name	Catalog Number	Quantity
Dicer siRNA Generation Kit	T510001	5 genes/50 transfections

For efficient transfection of DNA and high-level expression in cells:

Product Name	Catalog Number	Quantity
GenePORTER™ 2 Transfection Reagent	T202007	75 reactions (0.75 ml)
GenePORTER™ 2 Transfection Reagent	T202015	150 reactions (1.5 ml)
GenePORTER™ 2 Transfection Reagent	T202075	75 reactions (5 x 1.5 ml)

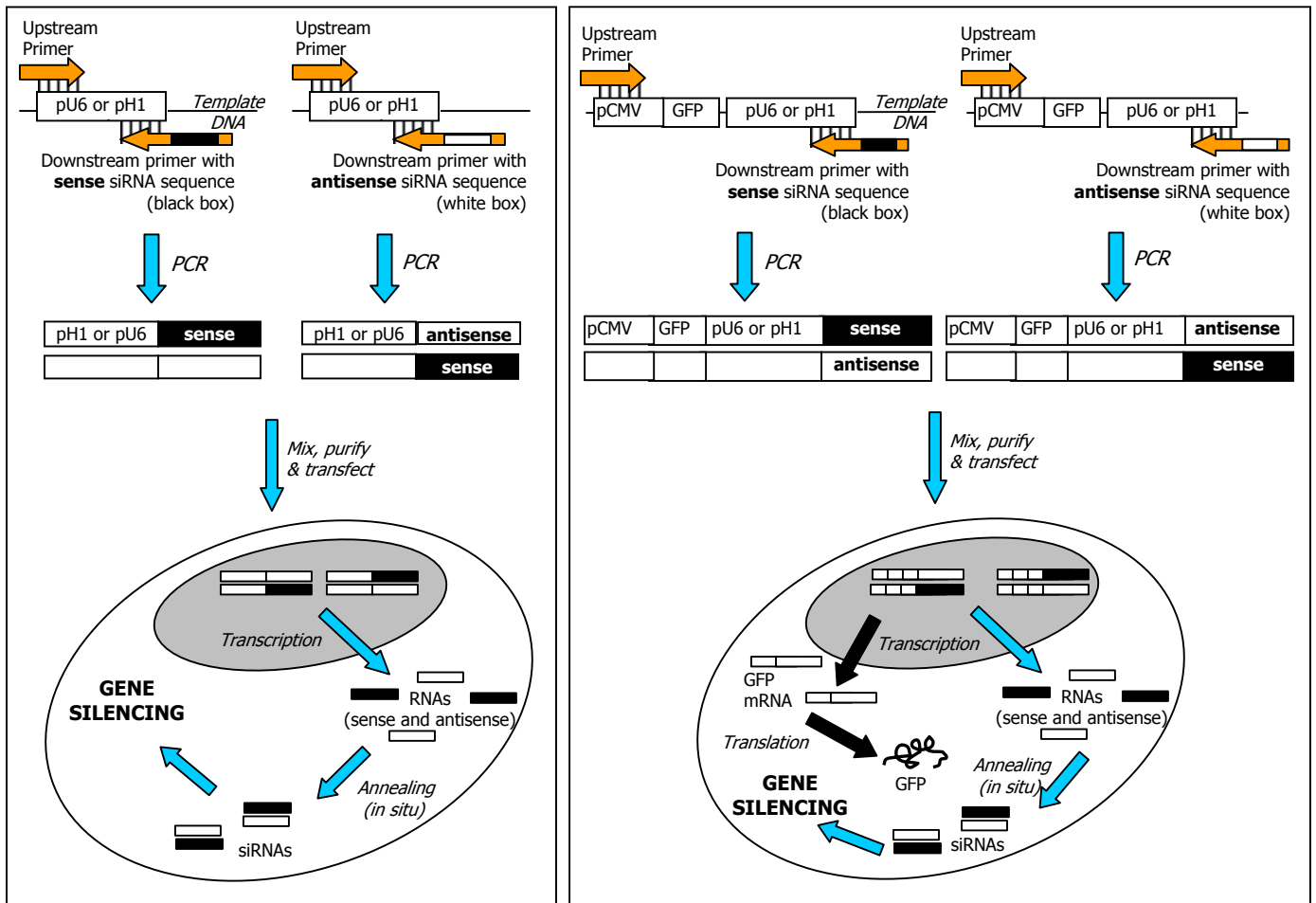
Introduction

Small interfering RNAs (siRNAs) are short double-stranded RNA molecules that facilitate potent and sequence-specific gene suppression via the mechanism of RNA interference (RNAi). When introduced into cultured mammalian cells, siRNAs facilitate the degradation of mRNA sequences to which they are homologous, thereby silencing the encoding gene.

The convenience of producing and using siRNAs has made them important tools for studying gene function. They can be synthesized *in vitro* and then introduced into cells directly; or they can be encoded in DNA expression vectors that are transfected into cells and subsequently expressed; or they can be produced by using the polymerase chain reaction (PCR) method and then transfected. Once transfected or expressed from a vector, siRNAs proceed to mediate RNAi-induced gene silencing.

The GTS GeneSilen[®] PCR kits provide a fast, convenient, and effective way to produce siRNAs *in vitro* without the need for cloning. The kits rely on the PCR technology to enable the production of sequence specific siRNAs. The desired siRNA expression cassettes are generated by a one step PCR, and are then transfected into cells. Subsequently, the cassettes are transcribed to produce sense and anti-sense siRNAs, which anneal *in situ* to form the duplex siRNAs that are effective for gene silencing.

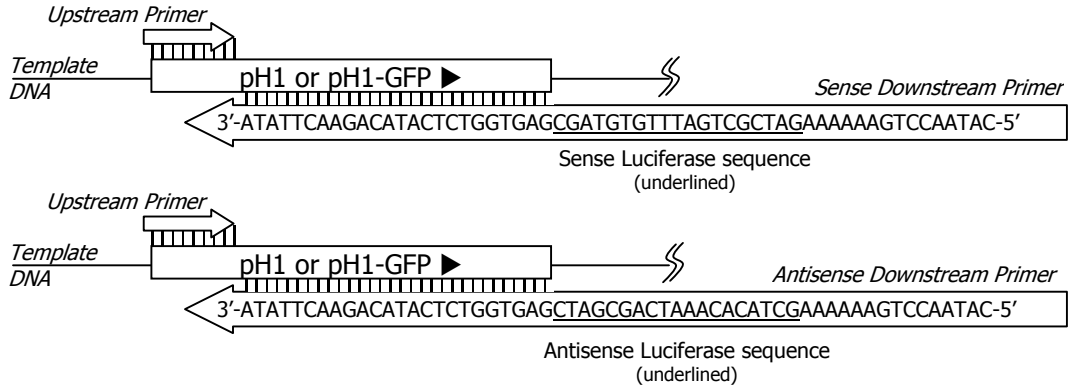
The following diagrams illustrate how the GeneSilen[®] PCR kits work:



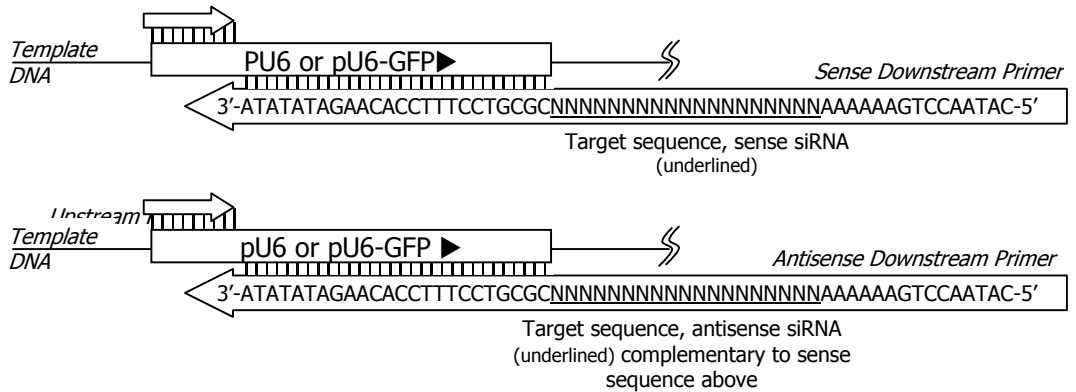
METHODS AND PROCEDURES

EXAMPLE: The following sequences illustrate the design of primers that produce siRNAs against the Luciferase gene.

Luciferase 19-mer target sequence: 5' -GCTACACAAATCAGCGATC-3'

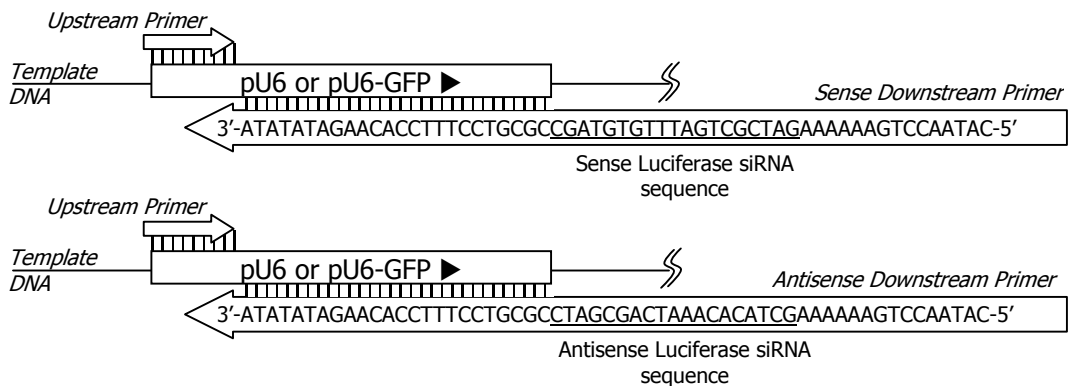


1.1.2. Downstream Primer Design for U6 or U6-GFP templates:



EXAMPLE: The following sequences illustrate the design of primers that produce siRNAs against the Luciferase gene.

Luciferase 19-mer target sequence: 5' -GCTACACAAATCAGCGATC-3'



METHODS AND PROCEDURES

1.2. PCR Amplification Reaction

- 1.2.1. Prepare a 100 μ l reaction mix by adding the following components together; *make sure to add the water first.*

82-x μ l	dd H ₂ O
10 μ l	PCR buffer (x10)
1 μ l	10 mM dNTP
5 μ l	template (50 ng)
1 μ l	upstream primer (1 μ g/ μ l)
1 μ l	downstream primer (1 μ g/ μ l)
x μ l	DNA polymerase (use supplier-recommended amount)
100 μ l	Total reaction volume

- 1.2.2. Run the PCR using the following recommended parameters:

94°C	3 min	
94°C	30 seconds	} x 35
55°C	30 seconds	
72°C	1 min/kb	
72°C	5–10 min	
4°C	Up to 24 hrs.	

1.3. Purification of PCR Fragments

- 1.3.1. Confirm the size and purity of PCR fragments by running 5 μ l on an agarose gel:

PCR Fragment Type	Size of PCR Fragment	% agarose gel to use
H1	153 bp	3%
U6	319 bp	1-3%
H1-GFP	1845 bp	1%
U6-GFP	2135 bp	1%

- 1.3.2. Mix the same amount of sense and antisense PCR fragments. The purification method needed will depend on how many impurities exist in the final PCR product; generally, we recommend using the following purification methods:

Purity of PCR Product	Purification Method Recommended
Single band	Buffer exchange or DNA purification methods available include ethanol precipitation ⁱ , DNA purification resins and/or spin columns. Many of these kits are available commercially
Multiple bands	Gel purification to extract the specific PCR fragment needed are suitable for this purpose. Use a commercially available kit or a common molecular biology protocol.

- 1.3.3. Reconfirm the purity of the PCR fragments by running a small sample on the appropriate agarose gel as recommended in Section 1.3.1). To obtain an accurate concentration of the purified PCR fragments, we recommend taking a spectrophotometric measurement of a diluted sample at OD₂₆₀.

2. Transfection of PCR Fragments Into Cells

The following siRNA transfection protocols are optimized for use with the GeneSilencer® siRNA Transfection Reagent. To obtain the GeneSilencer siRNA Transfection Reagent from Gene Therapy Systems, Inc., use any of the following catalog numbers.

Product Name	Catalog Number	Quantity
GeneSilencer® siRNA Transfection Reagent	T500750	200 reactions (0.75 ml)
GeneSilencer® siRNA Transfection Reagent	T505750	5 x 200 reactions (5 x 0.75 ml)
GeneSilencer® 96 Titration Plate	T500960	1 x 96 well plate
GeneSilencer® 96 Titration Plate	T504960	4 x 96 well plates
GeneSilencer® 96 Standard Plate High	T500961	1 x 96 well plate
GeneSilencer® 96 Standard Plate High	T504961	4 x 96 well plate
GeneSilencer® 96 Standard Plate Low	T500962	1 x 96 well plate
GeneSilencer® 96 Standard Plate Low	T504962	4 x 96 well plate

2.1. Transfection of Adherent Cells

- 2.1.1. The day before transfection, plate cells so that they will be 50-70% confluent on the day of transfection.
- 2.1.2. Prepare the GeneSilencer™ reagent by diluting in serum free medium according to Table 1 below.

Table 1: GeneSilencer™ Dilutions For Adherent Cells

Tissue Culture Plate or Dish Type	GeneSilencer™ Reagent (μl) + Serum Free Medium (μl) per well
96 wells	1.0 + 25
48 wells	1.75 + 25
24 wells	3.5 + 25
6 wells	5.0 + 25

- 2.1.3. Prepare the siRNA solution by first mixing siRNA Diluent and serum free medium (SFM) according to Table 2 below; *avoid vortexing the siRNA/Diluent mix*. Use the Diluent/SFM mix to dilute the recommended amount of siRNA in Table 2. Mix well by pipetting up and down several times. Incubate at Room Temperature for 5 minutes.

Table 2: siRNA Dilutions For Adherent Cells

Tissue Culture plate type	Recommended Amount of siRNA to use (ng) per well	siRNA Diluent (μl)+ Serum Free Medium (μl) per well	Final transfection volume (μl)
96 wells	50	2.5 + 15	100
48 wells	100	5.0 + 15	200
24 wells	200	10.0 + 15	500
6 wells	1,000	25.0 + 15	1,000

- 2.1.4. Add the siRNA solution from Step 2.1.3 to the diluted GeneSilencer solution in Step 2.1.2. Incubate at Room Temperature for 5 minutes to allow the siRNA/lipid complexes to form.

NOTE

You can incubate the siRNA/GeneSilencer™ mix for longer than 5 minutes, but make sure not to exceed 30 minutes in order to maintain maximum siRNA transfection efficiency.

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- 2.1.5. Add the siRNA/GeneSilencer™ mix to cells growing in serum-containing medium. Incubate at 37°C for 24 hours. See Table 2 for final transfection volume.
- 2.1.6. Add fresh tissue culture medium to growing cells as needed. Most RNA interference can be detected within 24 to 72 hours post transfection.

2.2. Transfection of Suspension Cells

- 2.2.1. The day before transfection, split the cells as necessary to optimize their health and achieve log-growth by transfection time.
- 2.2.2. Prepare the GeneSilencer™ reagent by diluting in serum free medium according to Table 3 below.

Table 3: GeneSilencer™ Dilutions For Suspension Cells

Tissue Culture plate type	GeneSilencer™ Reagent (μl) + Serum Free Medium (μl) per well
96 wells	1.0 + 25
48 wells	1.75 + 25
24 wells	3.5 + 25
6 wells	5.0 + 25

- 2.2.3. Prepare the siRNA solution by first mixing siRNA Diluent and serum free medium (SFM) according to Table 4 below; *avoid vortexing the siRNA Diluent mix*. Use the Diluent/SFM mix to dilute the recommended amount of siRNA in Table 4. Mix well by pipetting up and down several times. Incubate at Room Temperature for 5 minutes.

Table 4: siRNA Dilutions For Suspension Cells

Tissue Culture plate or dish type	Recommended Amount of siRNA to use (ng) per well	SiRNA Diluent (μl) + serum free medium (μl) per well
96 wells	50	2.5 + 15
48 wells	100	5.0 + 15
24 wells	200	10.0 + 15
6 wells	1,000	25.0 + 15

- 2.2.4. Add the siRNA solution from Step 2.2.3 to the diluted GeneSilencer® solution in Step 2.2.2. Incubate at Room Temperature for 5 minutes to allow the siRNA/lipid complexes to form.

NOTE

You can incubate the siRNA/GeneSilencer™ mix for longer than 5 minutes, but make sure not to exceed 30 minutes in order to maintain maximum siRNA transfection efficiency.

- 2.2.5. While the siRNA/GeneSilencer mix is incubating, spin down the cells from Step 2.2.1, remove the growth medium, and then resuspend the cells in the appropriate growth medium (serum-free or serum-containing) to achieve a final cell density listed in Table 5 below.

METHODS AND PROCEDURES

2.2.6. Transfer resuspended cells to culture plates according to Table 5 below.

Table 5: Volume and Number of Cells to Transfer Into Culture Dishes.

Tissue Culture plate or dish type	Volume of resuspended cells to transfer to each well (ml)	Number of cells transferred to each well (approximate).
96 wells	0.1	1×10^5
48 wells	0.2	2×10^5
24 wells	0.5	5×10^5
6 wells	1.0	2×10^6

2.2.7. Add the siRNA/GeneSilencer™ mix to resuspended cells in Table 5 above. Gently mix the cells by pipetting up and down several times; this step is important to avoid cell clumping. Incubate at 37°C for 24 hours.

2.2.8. Add fresh tissue culture medium to growing cells as needed. Most RNA interference can be detected within 24 to 72 hours post transfection.

Quality Control

To assure proper performance of the GeneSilencer® PCR Kit components, upstream and downstream primer pairs that target the luciferase gene are used in conjunction with the provided plasmid templates (H1, U6, H1-GFP, and U6-GFP) to generate sense and antisense PCR fragments. The resulting siRNA PCR fragments are purified, and their sizes are verified by agarose gel electrophoresis. The siRNA PCR fragments are then co-transfected along with a Luciferase expression plasmid into COS cells using the GeneSilencer® siRNA Transfection Reagent (Catalog Number T500750). Twenty-four hours post transfection, a luciferase assay is performed to monitor overall levels of Luciferase gene expression and the degree of silencing. Final results are normalized using a total protein assay. Results are compared to positive and negative controls for effective and specific gene silencing.

Troubleshooting Guide

Problem Description	Possible Causes	Recommended Solutions
No PCR products.	Wrong cycling parameters or program used.	Check your PCR cycler for the correct cycling parameters or program used.
	Missing or bad PCR mix component.	Verify that all PCR reaction components have been added.
		Use new or fresh PCR reaction components to avoid expired or incompatible products.
	Downstream primers.	Make sure that the sequences of the downstream primers have been selected correctly.
If not done yet, verify or measure the concentration of the primers using a spectrophotometer.		
Poor or no gene silencing.	Unpurified or sub-optimal amount of PCR fragments used.	Unpurified PCR fragments contain many buffer and PCR contaminants that may interfere with effective transfection and silencing. Make sure you purify the PCR fragments, and check their concentration afterwards. Make sure that enough of the purified PCR fragments have been used to transfect into cells.
	Inefficient/wrong target sequence.	Review your target sequence choice and pick other ones if possible.
	Poor Transfection Efficiency.	Make sure you are using a fresh transfection reagent and that you have followed the product use instructions correctly. If you are using the GeneSilencer® siRNA Transfection Reagent, check the Troubleshooting section in Appendix of the GeneSilencer product manual for more helpful recommendations.

Contact Information

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For a complete list of GTS international distributors, visit our web site at <http://www.genlantis.com>

References

¹Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, Second Edition (Plainview, New York: Cold Spring Harbor Laboratory Press).

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1994). Current Protocols in Molecular Biology (New York: Greene Publishing Associates and Wiley-Interscience)
