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SequiTherm EXCEL™ II DNA Sequencing Kit-LC (for 25-41 cm gels)

Cat. No. SE9101LC and SED52100

The SequiTherm EXCEL™ II DNA Sequencing Kit-LC (for 25-41 cm gels) is optimized for use with LI-COR® model 4x00 and NEN® Global IR2™ automated DNA sequencing machines using fluorescent (IR)-labeled primers. This kit incorporates advances† that allow researchers to sequence a broad range of templates, including many difficult templates.[1] Examples of difficult templates include those containing inverted repeats, short, and long tandem repeats, regions of high GC or AT content, and PCR products. In addition, nonspecific background signal is effectively reduced, resulting in clearer, less ambiguous data. We provide two protocols, a standard cycle sequencing protocol, and a high-temperature isothermal (non cycle) protocol. For most DNA templates, users may choose either protocol based on convenience. Nevertheless, we recommend using the

Cat. #		Quantity
SequiTherm EXCEL™ II DNA Sequencing Kit-LC (for 25-41 cm gels) Contents		
The SequiTherm EXCEL™ II DNA Sequencing Kit-LC (for 25-41 cm gels) is available in a 100-reaction size. The kit contains the following reagents:		
SequiTherm EXCEL™ II DNA Polymerase 500 U (Storage Buffer: 50% glycerol solution containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.1 mM EDTA, 0.5% NP-40, 0.5% Tween® 20, and 1 mM dithiothreitol.)	@ 5 U/µl	100 µl
3.5X SequiTherm EXCEL™ II Sequencing Buffer		750 µl
SequiTherm EXCEL™ II-LC Termination Mix G for 25-41 cm gels (0.03 mM ddGTP; 45 µM each of dATP, dCTP, dTTP, and 7-deaza-dGTP*)		200 µl
SequiTherm EXCEL™ II-LC Termination Mix A for 25-41 cm gels (0.45 mM ddATP; 45 µM each of dATP, dCTP, dTTP, and 7-deaza-dGTP*)		200 µl
SequiTherm EXCEL™ II-LC Termination Mix T for 25-41 cm gels (0.45 mM ddTTP; 45 µM each of dATP, dCTP, dTTP, and 7-deaza-dGTP*)		200 µl
SequiTherm EXCEL™ II-LC Termination Mix C for 25-41 cm gels (0.3 mM ddCTP; 45 µM each of dATP, dCTP, dTTP, and 7-deaza-dGTP*)		200 µl
Control Template (pSAD2)	(2 pmol @ 100 fmol/µl)	20 µl
Stop/Loading Buffer (95% formamide, 10 mM EDTA, 0.1% Basic Fuchsin, and 0.01% Bromophenol Blue [final pH 9])		1.5 ml
Sterile Deionized Water		1.5 ml

**7-deaza-dGTP is licensed from Boehringer Mannheim GmbH for research purposes only under U.S. patent no. 4,804,748.*

†Covered by patents and pending applications in the U.S. and other countries that are either assigned to or exclusively licensed to Epicentre.

isothermal protocol for those extremely difficult templates that produce unsatisfactory results with cycle sequencing.

Note: *The SequiTherm EXCEL II Termination Mixes included in this kit have been optimized for enhanced signal intensities in the range of 1-1,000 bases as commonly resolved on 25- to 41-cm long gels. Users of 66-cm gels can achieve read lengths of 1,000-1,400 bases by using the SequiTherm EXCEL II DNA Sequencing Kit-LC (for 66-cm gels).*

SequiTherm EXCEL II DNA Polymerase is also available as a stand-alone enzyme, sufficient for 100 sequencing reactions. Cat. No. SED52100.

Stop/Loading Buffer: The Stop/Loading Buffer supplied in this kit contains basic fuchsin dye, which is compatible with both 700- and 800-nm detection channels of LI-COR DNA sequencing machines. Store the Stop/Loading Buffer in the dark, as the basic fuchsin dye is sensitive to light. The Stop/Loading Buffer also contains a trace amount (0.01%) of bromophenol blue dye to aid in lane/well definition if the gel is being stagger-loaded. This amount of bromophenol blue dye will not interfere with signal detection when used in conjunction with IRD700 labels. Stop/Loading Buffers containing xylene cyanol are compatible with 800-nm channel detection, but will interfere with 700-nm channel detection.

Related Products: The following products are also available:

– SequiTherm EXCEL™ II DNA Sequencing Kit-LC (for 66-cm gels)

– EZ-Tn5™ Transposon Tools

– HyperMu™ Transposon Tools

Store the SequiTherm EXCEL™ II DNA Sequencing Kit-LC (for 25-41 cm gels) at –20°C in a freezer without a defrost cycle.

Notes on Sequencing

Handling Fluorescent (IRD)-Labeled Primers: IRD-labeled primers are sensitive to visible light. To avoid extended exposure of IRD-labeled primers to light: 1) keep primer stock tubes stored in the dark; 2) wrap primer stock tubes in foil for use on the benchtop; and 3) load final reaction products onto the sequencing gel as soon after completion of the reaction as possible or store at –20°C immediately. Individual reaction tubes (Premix and Termination Mix tubes) do not need to be wrapped in foil or kept in the dark while the reaction components are being aliquoted and mixed. Thermal cyclers do not need to be kept in the dark during cycling.

Custom Primers: For sequencing, we strongly recommend using oligonucleotides at least 20 bases in length with approximately 50% GC content. For primers shorter than 20 bases, or primers with less than 50% GC content, add a 30-second annealing step to the cycle program between the 95°C heat-denaturation step and the 70°C DNA synthesis step. Use 50°C as the annealing temperature for initial experiments with such primers. Alternatively, users can choose the annealing temperature based on the T_m(s) of the particular primer(s) in the experiment (see below).[2,3]

DNA Template Quality: The quality of sequence obtained with the SequiTherm EXCEL II DNA Sequencing Kit-LC (for 25-41 cm gels) will depend on the quality of the DNA template. Templates that give unsatisfactory results can usually be cleaned up by ethanol precipitation, or by phenol: chloroform extraction followed by ethanol precipitation. For best results, remove excess primers and unincorporated nucleotides from PCR reactions before using PCR products as templates in sequencing reactions. When preparing DNA from small cultures or single colonies, the alkaline lysis method[4] is recommended instead of lysis by boiling,[4] which introduces high levels of detergent that interfere with DNA synthesis.

EDTA Concentration: SequiTherm EXCEL II DNA Polymerase requires Mg^{2+} and may be inhibited by concentrations of EDTA >1 mM.

Calculating T_m: See references 2 and 3 for guidelines on calculating the T_m of oligonucleotide primers. However, the "4+2 Rule" ([4°C x #C+G's in the oligo] + [2°C x #A+T's in the oligo] will usually give a close enough estimation of the T_m for sequencing purposes.

Ethanol Precipitation Prior to Electrophoresis: Researchers may concentrate or pool the products of their sequencing reactions by precipitating with ethanol before loading the samples on a polyacrylamide gel.

Estimating Molar Amounts of Primer: To estimate the weight of 1 pmol of oligonucleotide, **multiply the number of nucleotides by 0.33 ng**.
For example, 1 pmol of a 24-mer oligonucleotide is: (24 x 0.33 ng = 7.92 ng).

Estimating Molar Amounts of Template: To estimate the weight of 100 fmol of double-stranded DNA template, **multiply the number of kilobases by 66 ng**.
For example, 100 fmol of a 2.7-kb plasmid is: (2.7 x 66 ng = 178 ng). See the table below for approximate amount of template required (in nanograms) for DNA sequencing.

Template Required	Template Size (insert + vector)				
	1 kb	3 kb	5 kb	7 kb	9 kb
50 fmol	33 ng	100 ng	165 ng	230 ng	300 ng
100 fmol	66 ng	200 ng	330 ng	460 ng	600 ng
150 fmol	100 ng	300 ng	500 ng	700 ng	900 ng
200 fmol	135 ng	400 ng	660 ng	930 ng	1,200 ng
250 fmol	165 ng	500 ng	830 ng	1,200 ng	1,550 ng
300 fmol	200 ng	600 ng	1,000 ng	1,400 ng	1,800 ng

[†]*pSAD2 provided courtesy of Dr. Grant McFadden, Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada.*

Cycle Sequencing pSAD2 Control DNA

The control template, pSAD2†, is a pUC-based clone containing an insert comprising a 150-base inverted repeat capable of forming a 75-basepair hairpin/cruciform structure. Several variations of this structure form at the temperatures commonly used in cycle sequencing reactions. When most DNA polymerases encounter these structures, the enzymes are unable to disrupt the base-paired structures and pause or terminate the primer extension, resulting in 4 lane stops and extraneous background signal.

Fig. 1 shows cycle sequencing data generated from the pSAD2 problematic region using an IRD800-labeled M13 forward primer. Clear sequence in the region starting at AAAA (+73 from the end of the M13 primer) and extending through the TTTTTTT (+122 from the end of the M13 primer) indicates that the SequiTherm EXCEL II DNA Sequencing Kit-LC is resolving the hairpin structure. Comparable data are obtained using the M13 reverse primer. Analysis of the sequencing data produced beyond base +122, however, shows that the reaction products form stable secondary structures in 8 M urea sequencing gels. These artifacts form only during electrophoresis and are characteristic of this specific control template. Presence of these gel artifacts do not indicate improper functioning of the kit.

Figure 1. Cycle Sequencing pSAD2. Lanes were loaded in the order G, A, T, C on a LI-COR model 4000 DNA Sequencer.

The kit is functioning properly if the sequence of pSAD2 between +73 and +122 is resolved using a cycle sequencing reaction.

Cycle Sequencing Protocol

This protocol describes the use of the SequiTherm EXCEL II DNA Sequencing Kit-LC (for 25-41 cm gels) with a LI-COR/NEN automated DNA sequencer and a single-labeled primer. If ambiguous or incomplete sequence data are obtained from a cycle sequencing reaction, then performing an isothermal sequencing reaction may generate additional or confirmatory information (see page 5). Reaction products are resolved for G, A, T, and C reactions in four lanes.

Protocol

1. Thaw the reagents listed in step 2 on ice and mix thoroughly.

   Note: *It is critical to mix all components by vortexing to ensure uniform composition of the solutions before dispensing.*

2. Combine the following components (total volume of 17 µl) in a 0.5-ml microfuge tube labeled "Premix":

x µl	deionized water
2 µl	(1-2 pmol) labeled primer(e.g., 2 pmol IRD800-labeled primer)
100-250 fmol	DNA template (1 µl of supplied pSAD2 control DNA)
7.2 µl	3.5X SequiTherm EXCEL II Sequencing Buffer
1 µl	SequiTherm EXCEL II DNA Polymerase (5 U/µl)
17 µl	Total reaction volume

3. For each template, label four 0.5-ml microfuge tubes with G, A, T, or C and place on ice. Then add:

   2 µl of SequiTherm EXCEL II-LC Termination Mix G for 25-41 cm gels to the G tube.
   2 µl of SequiTherm EXCEL II-LC Termination Mix A for 25-41 cm gels to the A tube.
   2 µl of SequiTherm EXCEL II-LC Termination Mix T for 25-41 cm gels to the T tube.
   2 µl of SequiTherm EXCEL II-LC Termination Mix C for 25-41 cm gels to the C tube.
4. On ice, add 4 µl of the Premix to each of the four tubes of Termination Mix; mix thoroughly.
5. Overlay each reaction with mineral oil.
6. Spin the tubes briefly in a microcentrifuge to separate the mineral oil from the reaction.
7. Heat the reactions for 5 minutes at 95°C to denature the template.
8. Perform 30 cycles using the following parameters: (See Important Note below)
   — 30 seconds at 95°C
   — 15 seconds at 50°C
   — 1 minute at 70°C
9. Add 3 µl of Stop/Loading Buffer to each reaction upon completion and either proceed with electrophoresis or store at –20°C. Reaction products may be stored at –20°C for several months. Alternatively, reactions may be held at 4°C overnight in the thermal cycler before adding the Stop/Loading Buffer.

Important Note

Custom primers may or may not require the use of an annealing step in the cycling profile. If the calculated annealing temperature of a primer is 65°C or higher, then a two-step cycling profile consisting of 30 seconds at 95°C and 1 minute at 70°C is recommended.

***Contact LI-COR for information on using two differentially labeled primers in a single reaction.*

Gel Electrophoresis

1. Centrifuge tubes briefly in a microcentrifuge to separate the mineral oil from the reaction.
2. Heat the reaction tubes for 3-5 minutes at ≥70°C to denature the samples; place on ice.
3. Load 1-2 µl per well onto a sequencing gel. Users can pipet an aliquot of each sample onto Parafilm® M. The mineral oil will adhere to the film, minimizing oil carryover and tip clogging.

Isothermal Sequencing Protocol

This protocol describes the use of the SequiTherm EXCEL II DNA Sequencing Kit-LC (for 25-41 cm gels) with a LI-COR/NEN automated DNA sequencer and a single-labeled primer** (see Fig. 2). Although users will resolve most templates using either the cycle or isothermal protocol, we recommend using the isothermal protocol with difficult templates that produce unsatisfactory results with cycle sequencing. Examples of difficult templates include those containing inverted repeats, short, and long tandem repeats, regions of high GC or AT content, and PCR products. Reaction products are resolved for G, A, T, and C reactions in four lanes.

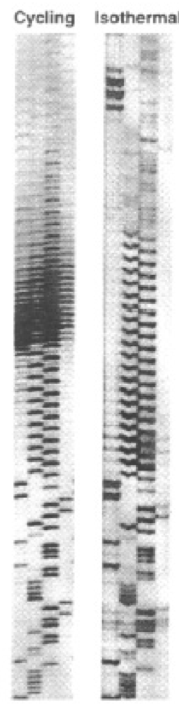

Figure 2. Cycle and Isothermal Sequencing of a dinucleotide repeat. Lanes were loaded in the order G, A, T, C on a LI-COR model 4000 DNA Sequencer.

Protocol

The optimal length of time for denaturation, annealing and extension will vary with individual templates (see Note, page 7). If the template does not contain the difficult regions mentioned above, users may perform the reactions using the lowest template recommendations (e.g., 600-800 fmol of template in Step 2) and follow the shorter recommended times for denaturation, annealing and extension (e.g., 1 minute to denature the template in Step 7). Conversely, when analyzing difficult templates, use more DNA (e.g., 800-1,000 fmol, Step 2), and follow the longer recommended times for denaturation, annealing, and extension (e.g., 5 minutes to denature the template in Step 7). Reactions may be performed using either a thermal cycler or water baths.

1. Thaw the reagents listed in step 2 on ice and mix thoroughly.

Note: *It is critical to mix all components by vortexing to ensure uniform composition of the solutions before dispensing.*

2. Combine the following components (total volume of 17 µl) in a 0.5-ml microfuge tube labeled "Premix":

x	µl	deionized water
2	µl	2 pmol IR-labeled primer
600-1000	fmol	DNA template
7.2	µl	3.5X SequiTherm EXCEL II Sequencing Buffer
1	µl	SequiTherm EXCEL II DNA Polymerase (5 U/µl)
17	µl	Total reaction volume

3. For each template, label four 0.5-ml microfuge tubes with G, A, T, or C and place on ice. Then add:

   2 µl of SequiTherm EXCEL II-LC Termination Mix G for 25-41 cm gels to the G tube.

   2 µl of SequiTherm EXCEL II-LC Termination Mix A for 25-41 cm gels to the A tube.

   2 µl of SequiTherm EXCEL II-LC Termination Mix T for 25-41 cm gels to the T tube.

   2 µl of SequiTherm EXCEL II-LC Termination Mix C for 25-41 cm gels to the C tube.

4. On ice, add 4 µl of the Premix to each of the four tubes of Termination Mix; mix thoroughly.
5. Overlay each reaction with mineral oil.
6. Centrifuge the tubes briefly in a microcentrifuge to separate the mineral oil from the reaction.
7. Heat the reactions for 1-5 minutes at 95°C to denature the template (see Note above).
8. Transfer the reactions to room temperature for 1-5 minutes to anneal the primer (see Note above).
9. Incubate the reactions at 65°C-70°C for 2-5 minutes to extend the annealed primer (see Note above).
10. Repeat Steps 7, 8, and 9 one more time.
11. Add 3 µl of Stop/Loading Buffer to each reaction upon completion of the second cycle and store at –20°C. Reaction products may be stored at –20°C for several months. Alternatively, reactions may be held at 4°C overnight before adding the Stop/Loading Buffer.

Gel Electrophoresis

1. Centrifuge tubes briefly in a microcentrifuge to separate the mineral oil from the reaction.
2. Heat the reaction tubes for 3-5 minutes at ≥70°C to denature the samples; place on ice.
3. Load 1-3 µl per well onto a sequencing gel. Users can pipet an aliquot of each sample onto Parafilm M. The mineral oil will adhere to the film, minimizing oil carryover and tip clogging.

Notes on Isothermal Sequencing

Denaturation: Denaturation occurs during the length of time outlined in Step 7. Nevertheless, the timing is not critical and templates may be incubated at 95°C for up to 30 minutes. A boiling-water bath may be used to denature some templates for sequencing provided incubation times do not exceed 1 minute. We do not recommend, however, using a boiling-water bath to denature templates containing regions difficult to sequence.

Annealing: Annealing of the primer to the template occurs during the length of time outlined in Step 8. Nevertheless, the timing is not critical, and may proceed up to 4 hours at room temperature. Templates containing regions difficult to sequence may require additional optimization. For these templates, following denaturation, slowly decrease the temperature at a rate of approximately 0.2°C per second to a few degrees just below or equal to the calculated melting temperature of the primer. (Users may achieve this with some thermal cyclers by turning off the thermal cycler after denaturation and monitoring the block temperature until the block cools slowly to the appropriate temperature.) Following annealing, proceed with Step 9 (also, see below).

Extension: Extension of the primer generally occurs during the length of time outlined in Step 9. The timing though, is not critical and may proceed up to 4 hours at 65°C-70°C. Templates containing regions difficult to sequence may also require optimization of the extension temperature depending on the nucleotide composition of the difficult region. For example, homopolymeric A or T regions or long $(AT)_n$ repeats may require extension temperatures of 60°C or lower. Homopolymeric G or C regions or long $(GC)_n$ repeats may require extension temperatures of 70°C or higher.

Fig. 2 shows the sequence ladder of a template containing a dinucleotide repeat (a difficult template that is recalcitrant to cycle sequencing) generated with both the cycling (page 4) and isothermal (page 5) protocols. Note that although using the isothermal protocol resolved the dinucleotide repeat, the background signal of the ladder before the repeat region is greater than that observed when using the cycling protocol.

Troubleshooting Sequencing Reactions

In the event of difficulties, sequencing the control template pSAD2 (see page 3) will confirm the proper functioning of the kit and aid in diagnosis. Possible problems and potential solutions are listed below.

Faint bands or no bands present on gel

1) **Insufficient template or primer.** Quantitate the DNA template by using electrophoresis or fluorescence and confirm the primer concentration.
2) **Impure template.** Purify problem templates by ethanol precipitation or phenol extraction followed by ethanol precipitation. Purify PCR products to remove excess primers, dNTPs, and secondary products.
3) **Poor annealing of primer to template.** Verify the sequence and orientation of the primer. Add an annealing step to the cycle protocol. We recommend 50°C as an initial annealing temperature. However, the optimal annealing temperature of the primer is at or slightly below the T_m.
4) **Evaporation of reactions.** The reaction must be covered with mineral oil before cycling. This may be helpful even in "hot-top" cyclers. Centrifuge the reaction before cycling to assure a good mineral oil seal.
5) **Sample loading problems.** Centrifuge the sample before loading to get good separation of oil and aqueous layers. Load by pipetting the sample from the lower, aqueous layer. Also, make sure to denature the samples before loading.
6) **Inactive polymerase.** Repeat the reactions using fresh polymerase.
7) **Inaccessible primer binding site.** Increase accessibility by nicking or linearizing the template DNA.
8) **Degraded IR label on primer.** Use fresh primer stock. Always store and handle primers as recommended.

Bands at the same position in all four lanes

1) **Impure template.** Purify problem templates by ethanol precipitation, or phenol extraction followed by ethanol precipitation. Purify PCR products to remove excess primers, dNTPs, and secondary products.
2) **Poor template quality.** The template may contain pyrimidine dimers, or may be excessively nicked. Prepare new template, avoiding UV irradiation.
3) **Cycler variability.** Cycle times may need to be modified for different thermal cyclers.
4) **Sample evaporation.** Cover the reaction with mineral oil before cycling. This may be helpful even in "hot-top" cyclers. Centrifuge the reaction before cycling to ensure a good mineral oil seal.
5) **Hybridization of primer to secondary sites on the template.** Sequence with a different primer. If using a separate annealing step, raise the annealing temperature.
6) **Contamination with primers that are less than full-length.** Purify full-length primer.
7) **Less active polymerase/sequencing buffers.** Repeat the reactions using new polymerase and buffers.

8) **Interference from Stop/Loading Buffer in 700-nm channel.** Use a Stop/Loading Buffer containing basic fuchsin instead of xylene cyanol.

Anomalous spacing of bands/missing bands

1) **Sequence compression due to secondary structure formation in DNA during electrophoresis.** Increase the electrophoresis temperature. Prepare the gel containing 40% formamide. Try sequencing the complementary strand. Use a primer either closer to or farther from the target site.

Faint "shadow bands" or "ghost bands" present in more than one lane

1) **Secondary primer binding site on template.** Remove the annealing step or raise the annealing temperature to increase stringency, or redesign the primer.
2) **Contamination with primers that are less than full-length.** Purify full-length primer.
3) **More than one template present in the reaction.** Prepare new plasmid or phage DNA, starting from a single colony or plaque. Gel-purify PCR products to remove secondary reaction products.
4) **Impure template.** Purify problem templates by ethanol precipitation or phenol extraction followed by ethanol precipitation. Purify PCR products to remove excess primers, dNTPs, and secondary products.
5) **Poor template quality.** Template may contain pyrimidine dimers, or is excessively nicked. Prepare new template avoiding UV irradiation.
6) **Too many cycles.** If using greater than 30 cycles, reduce the cycle number to 30. If problems persist, try using as few as 15 cycles.
7) **Less active polymerase.** Repeat the reactions using new polymerase.

High background throughout lanes

1) **Impure template.** Purify problem templates by ethanol precipitation or phenol extraction followed by ethanol precipitation. Purify PCR products to remove excess primers, dNTPs, and secondary products.
2) **Gel electrophoresis problems.** Excessive boiling to denature may degrade the sample. Clean the plates to remove residual soap/silanizing agent, which can cause smearing. Decrease the temperature during electrophoresis.
3) **Polyethylene glycol in template.** Purify template by an additional ethanol precipitation.

Faint or no bands in random lanes

1) **Unevenness among cycler wells.** Repeat reactions in a different thermal cycler.
2) **Tubes not uniform.** Non-uniform tubes result in uneven heating in the thermal cycler. Purchase tubes from another supplier.
3) **Evaporation of reactions.** See page 9 under "Faint bands or no bands present on gel".

4) **Impure template.** Purify problem templates by ethanol precipitation or phenol extraction followed by ethanol precipitation. Purify PCR products to remove excess primers, dNTPs, and secondary products.

Short reads/faint or no bands in same lane in all templates

1) **ddNTP breakdown in the Termination Mix.** Repeat with fresh nucleotide mixes. Thaw reaction reagents on ice, never in a waterbath.

Diffuse bands throughout gel

1) **Excess salt in sample.** Ethanol precipitate and wash template with 70% ethanol.
2) **Electrophoresis temperature too high.** Decrease temperature of electrophoresis to 50°C.
3) **Residual soap or silanizing agent on gel plates.** Clean plates thoroughly before use.
4) **Improper gel formation.** Use fresh, high-quality reagents that are stored in the dark. Allow adequate time for polymerization of the gel. Pour the gel at room temperature, not in the cold.
5) **Incorrect buffer composition.** Use a buffer stock that does not contain a precipitate.
6) **Samples not denatured.** Denature according to protocol before loading. However, avoid excessive boiling that can degrade the sample.
7) **Debris in wells when loading.** Rinse wells before loading to remove urea and unpolymerized acrylamide.
8) **Autosequencer out of focus.** Run focus routines and verify proper focal point.

References:

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2. McGraw, R.A. *et al.*, (1990) *BioTechniques* **8**, 674.
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4. Sambrook, J. *et al.*, (1989) *Molecular Cloning: A Laboratory Manual (2nd ed.)*, New York: Cold Spring Harbor Laboratory Press.

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