LEXOGEN

Enabling complete transcriptome sequencing

TELO™ PRIME

Full mRNA – precise ends

Full-Length cDNA Amplification Kit

User Guide

Catalog Numbers:
013.04 (TeloPrime Full-Length cDNA Amplification Kit, 4 preps)
013.08 (TeloPrime Full-Length cDNA Amplification Kit, 8 preps)
013.24 (TeloPrime Full-Length cDNA Amplification Kit, 24 preps)
018.30 (TeloPrime PCR Add-on Kit, 30 rxn)

013UG022V0100

FOR RESEARCH USE ONLY. NOT INTENDED FOR DIAGNOSTIC OR THERAPEUTIC USE.
INFORMATION IN THIS DOCUMENT IS SUBJECT TO CHANGE WITHOUT NOTICE.
Lexogen does not assume any responsibility for errors that may appear in this document.

PATENTS AND TRADEMARKS
The TeloPrime Full-Length cDNA Amplification Kits are covered by issued and/or pending patents. TeloPrime is a trademark of Lexogen. Lexogen is a registered trademark (EU, CH, USA).
SYBR Green I is a registered trademark of Life Technologies, Inc. RNasin is a trademark of Promega Corporation, RNaseZap is a registered trademark of Ambion, Inc., Bioanalyzer is a trademark of Agilent Technologies, Inc.
All other brands and names contained in this user guide are the property of their respective owners.

Lexogen does not assume responsibility for violations or patent infringements that may occur with the use of its products.

LIABILITY AND LIMITED USE LABEL LICENSE: RESEARCH USE ONLY
This document is proprietary to Lexogen. The TeloPrime Full-Length cDNA Amplification Kits are intended for use in research and development only. They need to be handled by qualified and experienced personnel to ensure safety and proper use. Lexogen does not assume liability for any damage caused by the improper use or the failure to read and explicitly follow this user guide. Furthermore, Lexogen does not assume warranty for merchant-ability or suitability of the product for a particular purpose.
The purchase of the product does not convey the right to resell, distribute, further sublicense, repackage, or modify the product or any of its components. This document and its contents shall not be used or distributed for any other purpose and/or otherwise communicated, disclosed, or reproduced in any way without the prior written consent of Lexogen.

For information on purchasing additional rights or a license for use other than research, please contact Lexogen.

WARRANTY
Lexogen is committed to providing excellent products. Lexogen warrants that the product performs to the standards described in this user guide until the expiration date. Should this product fail to meet these standards due to any reason other than misuse, improper handling, or storage, Lexogen will replace the product free of charge or issue a credit for the purchase price. Lexogen does not provide any warranty if product components are replaced with substitutes. Under no circumstances shall the liability of this warranty exceed the purchase price of this product.

LITERATURE CITATION
When describing a procedure for publication using this product, please refer to it as Lexogen's TeloPrime Full-Length cDNA Amplification Kit.

We reserve the right to change, alter, or modify any product without notice to enhance its performance.

CONTACT INFORMATION

Lexogen GmbH
Campus Vienna Biocenter 5
1030 Vienna, Austria
www.lexogen.com
E-mail: info@lexogen.com

Support
E-mail: support@lexogen.com
Tel. +43 (0) 1 3451212-41
Fax. +43 (0) 1 3451212-99

Table of Contents

1. Overview 4
2. Kit Components and Storage Conditions 6
3. User-supplied Consumables and Equipment 7
4. Guidelines 7
5. Detailed Protocol 11
   - 5.1 Full-Length cDNA Synthesis 11
   - 5.2 Full-Length cDNA Amplification 14
6. Short Procedure 16
7. Appendix A: RNA Requirements 18
8. Appendix B: Calculation of the Endpoint PCR 20
9. Appendix C: Full-Length cDNA Amplification Quality Control . 21
10. Appendix D: Downstream Applications 22
11. Appendix E: Revision History 23

LEXOGEN

Enabling complete transcriptome sequencing

1. Overview

The TeloPrime Full-Length cDNA Amplification Kit is an all-in-one protocol for generating full-length cDNA from total RNA. Based on Lexogen´s unique Cap-Dependent Linker Ligation (CDLL) and long reverse transcription (long RT) technology, it is highly selective for full-length RNA molecules that are both capped and polyadenylated. TeloPrime amplified cDNA provides a very faithful representation of the mRNA transcriptome, empowering downstream applications such as NGS, cloning, or RACE (Appendix D). It enables the detection and correct quantification of splice variants and their true transcription start- and end-sites, in both short and long mRNA molecules.

In a first step, full-length cDNA synthesis is initiated by oligodT primed long reverse transcription. This immediately preserves the complete RNA sequence information in the cDNA before a cap selection is carried out. In addition a more stable RNA : cDNA hybrid is created that is maintained throughout post RT purification.

This double stranded RNA : cDNA hybrid is also important for the specificity of the subsequent Cap-Dependent Linker Ligation reaction. There a double-stranded adapter with a 5′ C overhang allows for an atypical base-pairing with the inverted G of the cap structure. By using a double-strand-specific ligase, the ligation will only take place if the cap is present and if the RT has really reached the 5′ end of the mRNA. No ligation will take place if no cap is present e.g., in degraded RNA (low RIN) or if the RT has terminated prematurely because of secondary structures.
Therefore the ligation of the 5′ linker tag to the 3′ end of the cDNA takes place in a highly cap-dependent manner. In the subsequent second-strand synthesis and purification steps all remaining background is eliminated and only 5′ tagged full-length cDNA is converted into full-length double stranded (ds) cDNA.

The full-length ds cDNA is then globally amplified in a PCR reaction using 5′ and 3′ tag specific PCR primers to provide enough material for downstream applications, such as RACE, cloning, sequencing, and library or probe generation. For in depth gene-specific analysis, Lexogen offers also a TeloPrime PCR Add-on Kit with additional 30 PCR reactions (Cat.No. 018.30) that can be adapted with gene-specific primers. Quantification and quality control of the final amplification product should be done using standard methods and is further discussed in Appendix C.

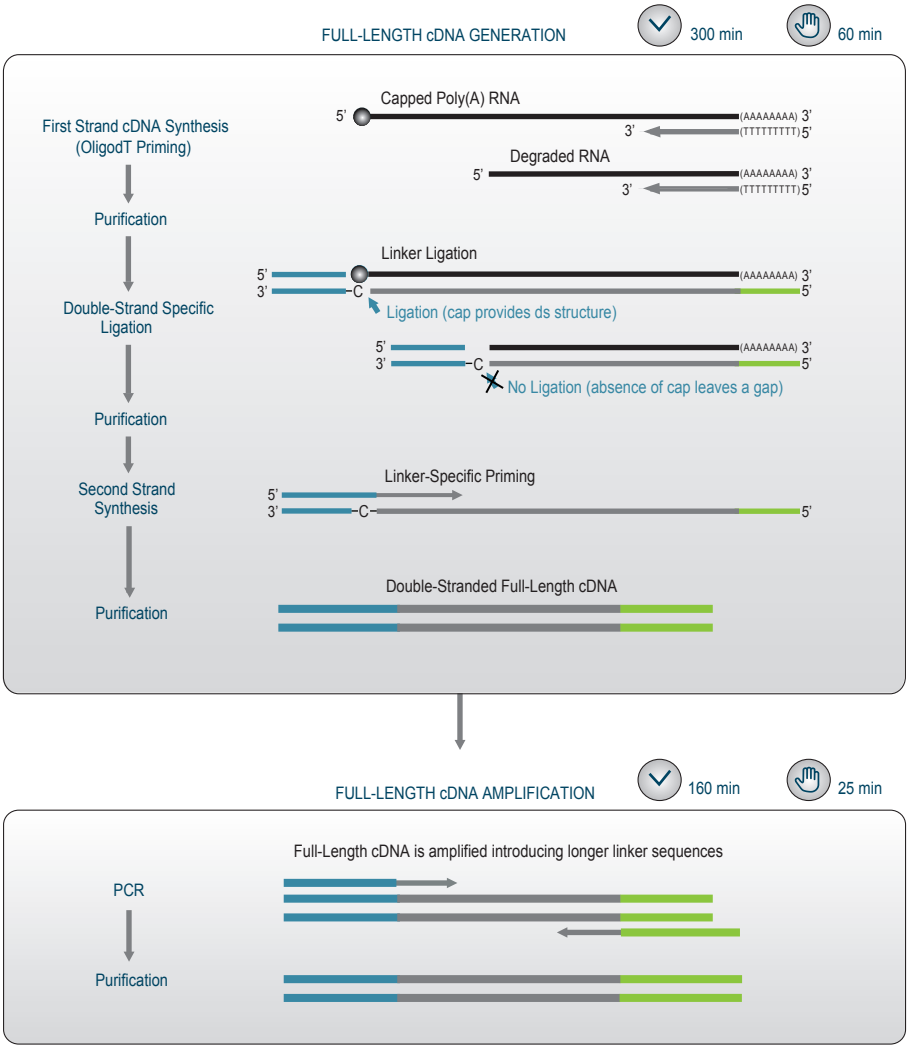


Figure 1. Schematic overview of the TeloPrime workflow.

2. Kit Components and Storage Conditions

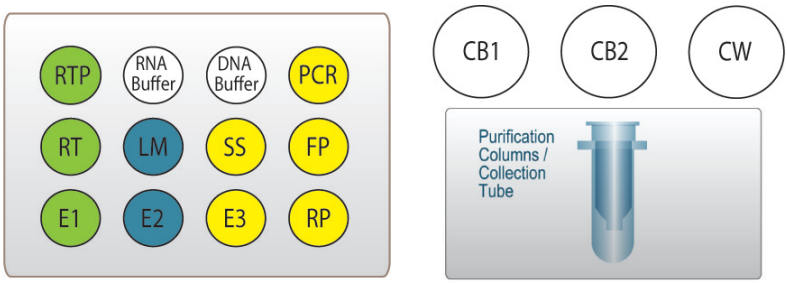


Figure 2. Location of kit contents.

Kit Components	Tube Label		Volume needed for [1]		Storage
		4 preps	8 preps	24 preps	
Reverse Transcription Primer	**RTP** ●	8.8 µl	17.6 µl	52.8 µl	-20 °C
Enzyme Mix 1	**E1** ●	8.8 µl	17.6 µl	52.8 µl	-20 °C
Reverse Transcription Mix	**RT** ●	17.6 µl	35.2 µl	105.6 µl	-20 °C
Enzyme Mix 2	**E2** ●	8.8 µl	17.6 µl	52.8 µl	-20 °C
Ligation Mix	**LM** ●	88 µl	176 µl	528 µl	-20 °C
Enzyme Mix 3	**E3** ●	8.8 µl	17.6 µl	52.8 µl	-20 °C
Second Strand Synthesis Mix	**SS** ●	26.4 µl	52.8 µl	154 µl	-20 °C
PCR Forward Primer	**FP** ●	8.8 µl	17.6 µl	52.8 µl	-20 °C
PCR Reverse Primer	**RP** ●	8.8 µl	17.6 µl	52.8 µl	-20 °C
PCR Mix	**PCR** ●	22 µl	44 µl	132 µl	-20 °C
RNA Buffer	**RNA Buffer** ○	220 µl	440 µl	1320 µl	-20 °C
DNA Buffer	**DNA Buffer** ○	220 µl	440 µl	1320 µl	-20 °C
Column Binding Buffer 1	**CB1** ○	2.11 ml	4.22 ml	12.67 ml	RT
Column Binding Buffer 2	**CB2** ○	1.41 ml	2.82 ml	8.45 ml	RT
Column Wash Buffer	**CW** ○	8.80 ml[2]	17.60 ml[2]	52.80 ml[2]	RT

[1] Including a 10 % surplus [2] Including ethanol (to be added by user)

Upon receiving the TeloPrime kit, remove the smaller inner box and store it in a -20 °C freezer. The rest of the kit components should be stored at room temperature and protected from light. Before use, check the contents of **CB1** ❍, **CB2** ❍, and **CW** ❍. If a white precipitate is visible, incubate at 37 °C until buffer components dissolve completely.

Cat. No. 0013.04 (4 preps): Add 8 ml absolute ethanol to **CW** ❍ and shake to combine.
Cat. No. 0013.08 (8 preps): Add 16 ml absolute ethanol to **CW** ❍ and shake to combine.
Cat. No. 0013.24 (24 preps): Add 48 ml absolute ethanol to **CW** ❍ and shake to combine.

3. User-supplied Consumables and Equipment

Check to ensure that you have all of the necessary materials and equipment before beginning with the protocol. All reagents, equipment, and labware must be free of nucleases and nucleic acid contamination.

Reagents

- Absolute ethanol, add to Column Wash Buffer (**CW** ❍).
- SYBR Green I (Life Technologies, Inc.).

Equipment

- Benchtop centrifuge (3,000 x g, rotor compatible with 96-well plates).
- Benchtop centrifuge (12,000 x g, rotor compatible with 1.5 ml and 2.0 ml micro-tubes).
- Calibrated single-channel pipettes for handling 1 µl to 1000 µl volumes.
- qPCR machine.
- Thermocycler.
- Vortex mixer.
- Ice bath or ice box, ice pellets, benchtop cooler (-20 °C for enzymes).

Optional equipment

- Automated microfluidic electrophoresis station (Agilent Technologies 2100 Bioanalyzer).
- Agarose gels, dyes, and electrophoresis rig.

Labware

- Suitable certified ribonuclease-free pipette tips (pipette tips with aerosol barriers recommended).
- 1.5 ml micro-tubes with cap, low binding, certified ribonuclease-free.
- 200 µl PCR tubes or 96-well plates and caps or sealing foil.

The complete set of materials, reagents, and labware necessary for quality control is not listed.

4. Guidelines

RNA Handling

- RNases are ubiquitous and special care should be taken throughout the procedure to avoid RNase contamination.
- Use commercial ribonuclease inhibitors (i.e. RNasin, Promega Corp.) to maintain RNA integrity when storing samples.
- Use a sterile and RNase-free workstation or laminar flow hood if available. Please note that RNases may still be present on sterile surfaces, and that autoclaving does not completely eliminate RNase contamination. Before starting cDNA synthesis, clean your work space, pipettes, and other equipment with RNase removal spray (such as RNaseZap, Ambion Inc.) as per the manufacturer's instructions.
- Protect all reagents and your RNA samples from RNases on your skin by wearing a clean lab coat and fresh gloves. Change gloves after making contact with equipment or surfaces outside of the RNase-free zone.
- Avoid speaking above opened tubes. Keep reagents closed when not in use to avoid airborne RNase contamination.

General

- In steps 2, 21, 30 and 34 of the TeloPrime protocol mastermixes of enzymes and reaction buffers should be prepared. When preparing mastermixes and when using multi-channel pipettes always include a 10 % surplus per reaction in order to have enough solution available for all reactions. All reagents of the TeloPrime kit include a 10 % surplus.
- Unless explicitly mentioned, all steps should be carried out at room temperature (RT) between 20 °C and 25 °C. Results may be negatively impacted if the protocol is performed at temperatures outside of this range. While reaction set-up is often performed at RT, incubation temperatures are explicitly defined, and must be strictly adhered to.
- To further increase reproducibility and to avoid cross contamination a centrifugation step should be performed after incubations at elevated temperatures and before removing the sealing foil from PCR plates or opening tubes.
- Ensure that adequate volumes of all reagents and the necessary equipment are available before beginning the protocol.
- Perform all pipetting steps with calibrated pipettes and always use fresh tips. Pipette carefully to avoid foaming as some solutions contain detergents.
- Thaw all necessary buffers at room temperature or as indicated in the preparation tables at the beginning of each step of the detailed protocol. Mix reagents well by vortexing or

pipetting repeatedly and centrifuge briefly with a benchtop centrifuge to collect contents before use.

- Keep enzyme mixes at -20 °C until right up before use or store in a -20 °C benchtop cooler.
- When mixing by pipetting, set the pipette to a larger volume. For example after adding 6 µl in step 4 use a pipette set to 15 µl or 20 µl to ensure proper mixing.
- Before you start, check all solutions for the formation of precipitate and if necessary, incubate at 37 °C until buffer components dissolve completely.
- If necessary the protocol can be stopped after each purification step and samples can be stored at -20 °C.

Pipetting and Handling of (Viscous) Solutions

- Enzyme mixes and Ligation Mix (**LM** ●) are viscous solutions which require care to pipette accurately. Quickly spin down the tubes to collect all liquid at the bottom of the tube. Be sure to pipette slowly and check the graduation marks on your pipette tips when removing an aliquot.
- When drawing up liquid, the tip should be dipped 3 to 5 mm below the surface of the liquid, always at a 90 degree angle. Do not dip in the tip any further as viscous solutions tend to stick to the outside of the pipette tip.
- Any residual liquid adhering to the tip should be removed by sliding the tip up the wall or edge of the tube from which the liquid was taken. Spin down the tube afterwards again to ensure that all liquid is collected at the bottom of the tube for further storage.
- When dispensing, the pipette should be held at a 45 degree angle, and the tip placed against the side of the receiving vessel.
- When pipetting liquids from bottles, take special care that only the sterile pipette tip touches the bottle opening to prevent introducing RNases or other contaminants. Tips are sterile, whereas the pipette itself is not. If necessary, tilt the bottle to bring the liquid closer to the opening and facilitate pipetting

Safety Information

Column Binding Buffer 1 (**CB1** ❍) and Column Binding Buffer 2 (**CB2** ❍) contain guanidine isothiocyanate, an irritant, which might also be present in the flow-through fractions. This chemical is harmful, contact with acids liberates very toxic gas (hydrogen cyanide).

Do not add bleach or acidic solutions directly to solutions or sample preparation waste that contains guanidine isothiocyanate.

Solutions containing isopropanol or ethanol are considered flammable. Use appropriate precautions when using these chemicals.

For your protection, always wear a laboratory coat, gloves, and safety glasses when handling these chemicals.

Dispose of the buffers and chemicals in appropriate waste containers.

Consult the appropriate Material Safety Data Sheets (MSDS), available at www.lexogen.com, and contact your Environmental Health and Safety department for proper work and disposal guidelines.

5. Detailed Protocol

5.1 Full-Length cDNA Synthesis

Preparation

First Strand Synthesis	Ligation	Second Strand Synthesis	Purification
RNA Buffer – thawed at RT **RT** – thawed at RT **RTP** – thawed at RT **E1** – keep on ice or at -20 °C	**LM** – thawed at RT **E2** – keep on ice or at -20 °C	**SS** – thawed at RT **E3** – keep on ice or at -20 °C	**CB1** – stored at RT **CB2** – stored at RT **CW** – stored at RT **RNA Buffer** – thawed at RT **DNA Buffer** – thawed at RT
Thermocycler 70 °C for 30 sec 37 °C for 1 min hold at 37 °C to add MM 37 °C for 2 min 46 °C for 50 min 10 °C ∞	Thermocycler 25 °C for 3 h 10 °C ∞	Thermocycler 98 °C for 90 sec 62 °C for 60 sec 72 °C for 5 min 25 °C ∞	Benchtop centrifuge set to 18 °C Purification Column Collection Tube

First Strand cDNA Synthesis – Reverse Transcription

RNA samples and the oligodT primer (**RTP** ●) are briefly heated to resolve secondary structures and promote efficient hybridization of the primer before a reverse transcription is performed. For information on appropriate amounts of total RNA input as well as RNA quantification and quality control see Appendix A (p.18).

1. Dilute 1 ng - 2 µg of total RNA to a volume of 12 µl with **RNA Buffer** ❍ and mix it with 2 µl of Reverse Transcription Primer (**RTP** ●).
2. Prepare Mastermix 1 containing 4 µl Reverse Transcription Mix (**RT** ●) and 2 µl Enzyme Mix 1 (**E1** ●) per reaction. Mix well.
3. Denature RNA / **RTP** ● mix using a thermocycler at 70 °C for 30 seconds, cool down to 37 °C, and incubate for 1 minute.
4. Leave the reaction on the thermocycler on hold and add 6 µl of Mastermix 1 to each reaction, mix by pipetting, and seal the tubes or plate.
5. Incubate at 37 °C for another 2 minutes, raise the temperature to 46 °C for 50 minutes, then hold at 10 °C. **NOTE:** Spin down before proceeding to the next step.

Purification

The cDNA : RNA hybrid is purified using silica columns to remove all reaction components.

6	Add a total of 160 µl Column Binding Buffer (**CB1** ❍) to the reaction, mix well, and transfer the solution to a Purification Column placed in a 2 ml Collection Tube. Centrifuge for 1 minute at 12,000 x g at 18 °C.
7	Apply 200 µl of Column Wash Buffer (**CW** ❍) to the column and centrifuge for 1 minute at 12,000 x g at 18 °C.
8	Repeat this washing step once (for a total of two washes).
9	Discard the flow-through. Centrifuge for 2 minutes at 12,000 x g at 18 °C to dry the column.
10	Transfer the column to a new 1.5 ml tube and apply 19 µl of **RNA Buffer** ❍ to the column. Incubate at room temperature for 1 minute and centrifuge for 2 minutes at 12,000 x g at 18 °C to elute the cDNA : RNA hybrid.
11	Transfer 18 µl of the eluted sample to a fresh PCR tube or plate.

Ligation

During this step the adapter is ligated to the cDNA in the hybrid by base-pairing of the 5´C to the cap structure of the RNA, using a double-strand specific ligase.

ATTENTION: Ligation Mix (**LM** ●) is a viscous solution and needs to be mixed thoroughly before use.

12	Prepare Mastermix 2 containing 20 µl of Ligation Mix (**LM** ●) and 2 µl of Enzyme Mix 2 (**E2** ●) per reaction. Mix well.
13	Add 22 µl of Mastermix 2 to each reaction. Mix well.
14	Incubate the reaction at 25 °C for 3 hours.

Purification

The ligated cDNA : RNA hybrid is purified using silica columns to remove all reaction components, including excess adapter.

15	Add a total of 320 µl Column Binding Buffer (**CB1** ❍) to the reaction, mix well, and transfer the solution to a Purification Column placed in a 2 ml Collection Tube. Centrifuge for 1 minute at 12,000 x g at 18 °C.
16	Apply 400 µl of Column Wash Buffer (**CW** ❍) to the column and centrifuge for 1 minute

at 12,000 x g at 18 °C.

17 Repeat this washing step once (for a total of two washes).

18 Discard the flow-through. Centrifuge for 2 minutes at 12,000 x g at 18 °C to dry the column.

19 Transfer the column to a new 1.5 ml tube and apply 14 µl of **RNA Buffer** ❍ to the column. Incubate at room temperature for 1 minute and centrifuge for 2 minutes at 12,000 x g at 18 °C to elute the cDNA : RNA hybrid.

20 Transfer 13 µl of the eluted sample to a fresh PCR tube or plate.

Second Strand Synthesis

The full-length cDNA is converted to dsDNA using the PCR Forward Primer (**FP**).

21 Prepare Mastermix 3 containing 6 µl of Second Strand Mix (**SS** ●) and 1 µl of Enzyme Mix 3 (**E3** ●) per reaction. Mix well.

22 Add 7 µl of Mastermix 3 to each reaction. Mix well.

23 Conduct one cycle of thermocycling with the following program: 98 °C for 90 seconds, 62 °C for 60 seconds, 72 °C for 5 minutes, hold at 25 °C.

Purification

The double-stranded fragments are purified to remove all reaction components and any potential single-stranded template switch background of the Reverse Transcription Primer.

24 Add a total of 160 µl Column Binding Buffer (**CB2** ❍) to the reaction, mix well, and transfer the solution to a Purification Column placed in a 2 ml Collection Tube. Centrifuge for 1 minute at 12,000 x g at 18 °C.

25 Apply 200 µl of Column Wash Buffer (**CW** ❍) to the column and centrifuge for 1 minute at 12,000 x g at 18 °C.

26 Repeat this washing step once (for a total of two washes).

27 Discard the flow-through. Centrifuge for 2 minutes at 12,000 x g at 18 °C to dry the column.

28 Transfer the column to a new 1.5 ml tube and apply 19 µl of **DNA Buffer** ❍ to the column. Incubate at room temperature for 1 minute and centrifuge for 2 minutes at 12,000 x g at 18 °C to elute the dsDNA.

29 Transfer 18 µl of the eluted sample to a fresh PCR tube or plate.

5.2 Full-Length cDNA Amplification

Preparation

PCR	Purification
FP – thawed at RT **RP** – thawed at RT **PCR** – thawed at RT **E3** – keep on ice or at -20 °C **SYBR Green I** -dilute 1:5000 in DMSO, at RT	**CB1** – stored at RT **CB2** – stored at RT **CW** – stored at RT **DNA Buffer** – thawed at RT
Thermocycler 98 °C for 30 sec 50 °C for 90 sec 72 °C for 5 min 98 °C for 30 sec 62 °C for 60 sec } xx 72 °C for 5 min } Appendix B, p.20 72 °C for 5 min 10 °C ∞	Benchtop centrifuge set to 18 °C Purification Column Collection Tube

qPCR

The full-length double stranded cDNAs are first amplified in a qPCR reaction using 3' and 5' end-specific primers (PCR Forward Primer (**FP** ●) and Reverse Primer (**RP** ●)) to determine the exact cycle number for the endpoint PCRs. If desired, **RP** ● and **FP** ● can be exchanged for a gene-specific primer (information on the primer concentrations are given in Appendix D, p.22).

ATTENTION: Please make sure to add SYBR Green I (not included in the kit) to a final concentration of 0.1 x. Higher concentrations will inhibit full-length amplification.

30 Prepare Mastermix 4 containing 5 µl of PCR Mix (**PCR** ●), 2 µl of the PCR Forward Primer (**FP** ●), 2 µl of the PCR Reverse Primer (**RP** ●), 1 µl of Enzyme Mix 3 (**E3** ●) and 1 µl of a 1 : 5000 SYBR Green I dilution (diluted in DMSO, final concentration in the PCR reaction must be 0.1 x).

31 Add 11 µl of Mastermix 4 to up to 9 µl sample. If less sample is used add H_2O or **DNA Buffer** ❍ to a final volume of 20 µl per reaction. Mix well. **NOTE:** If the template should be amplified in more than one PCR reaction use smaller aliquots.

32 Conduct 40 cycles of thermocycling with the following program: One cycle of 98 °C for 30 seconds, 50 °C for 90 seconds, 72 °C for 5 minutes, 39 cycles of 98 °C for 30 seconds, 62 °C for 60 seconds, 72 °C for 5 minutes, and a final extension at 72 °C for 5 minutes, hold at 25 °C.

33 Determine the fluorescence value of the plateau. Calculate at which cycle the fluorescence is at 80 % from the maximum (usually three cycles less than that needed to reach the plateau); this is the cycle number to be used for the endpoint PCR (see Appendix B, p.20)

Endpoint PCR

The endpoint PCRs are performed to generate enough material for any subsequent application (such as NGS sample preps, microarrays, or cDNA cloning). The exact determination of the cycle number in the qPCR prevents over- or undercycling, which could have negative effects on downstream applications (see Appendix B, p.20). Here, SYBRGreen I can be left out of the mix to maximize length.

34 Prepare Mastermix 5 containing 5 µl of PCR Mix (**PCR** ●), 2 µl of the PCR Forward Primer (**FP** ●), 2 µl of the PCR Reverse Primer (**RP** ●), and 1 µl of E3 Mix (**E3** ●) and 1 µl of H_2O or **DNA Buffer** ❍.

35 Add 10 µl Mastermix 5 to up to 9 µl of sample. If less sample is used add H_2O or **DNA Buffer** ❍ to a final volume of 20 µl per reaction. Mix well.

36 Conduct xx cycles (see Appendix B, p.20) of thermocycling with the following program: 98 °C for 45 seconds, 50 °C for 90 seconds, 72 °C for 5 minutes, xx cycles (as determined in step 33) of 98 °C for 30 seconds, 62 °C for 60 seconds, 72 °C for 5 minutes, and a final extension at 72 °C for 5 minutes, hold at 25 °C.

Purification

The amplified transcripts are purified from PCR components that can interfere with quantification and other downstream applications.

37 Add a total of 160 µl Column Binding Buffer (**CB2** ❍) to the reaction, mix well, and transfer the solution to a Purification Column placed in a 2 ml Collection Tube. Centrifuge for 1 minute at 12,000 x g at 18 °C.

38 Apply 200 µl of Column Wash Buffer (**CW** ❍) to the column and centrifuge for 1 minute at 12,000 x g at 18 °C.

39 Repeat this washing step once (for a total of two washes).

40 Discard the flow-through. Centrifuge for 2 minutes at 12,000 x g at 18 °C to dry the column.

41 Transfer the column to a new 1.5 ml tube and apply 21 µl of **DNA Buffer** ❍ to the column. Incubate at room temperature for 1 minute and centrifuge for 2 minutes at 12,000 x g at 18 °C to elute the dsDNA fragments.

42 At this point, the full-length cDNA amplification is finished and ready for quality control (Appendix C, p.21)

6. Short Procedure

All centrifugation steps are at 12,000 x g and 18 °C.

300 min	Full-Length cDNA Synthesis
☐ Dilute 1 ng to 2 µg total RNA in 12 µl **RNA Buffer** ○	
☐ Add 2 µl of **RTP** ● and mix well	
☐ Pre-mix 4 µl **RT** ● and 2 µl of **E1** ● per reaction	First Strand cDNA Synthesis
☐ Denature RNA / **RTP** mix for 30 sec at 70 °C, cool down to 37 °C and incubate for 1 min, hold at 37°C	
☐ Add 6 µl **RT** / **E1** mix per reaction, mix well	
☐ Incubate for 2 min at 37 °C and raise to 46 °C for 50 minutes	
☐ Add 160 µl of **CB1** ○, mix and apply to column, centrifuge 1 min	
☐☐ Add 200 µl of **CW** ○, centrifuge 1 min, repeat once	
☐ Discard flow-through, centrifuge 2 min	Purification
☐ Exchange Collection Tube with 1.5 ml tube	
☐ Add 19 µl **RNA Buffer** ○, incubate 1 min at RT, centrifuge 2 min	
☐ Transfer 18 µl of sample to new PCR tube/plate	
☐ Pre-mix 20 µl **LM** ● and 2 µl **E2** ● per reaction	Ligation
☐ Add 22 µl of **LM / E2** mix to each reaction	
☐ Incubate at 25 °C for 3 h	
☐ Add 320 µl of **CB1** ○, mix and apply to column, centrifuge 1 min	Purification
☐☐ Add 400 µl of **CW** ○, centrifuge 1 min, repeat once	
☐ Discard flow-through, centrifuge 2 min	
☐ Exchange Collection Tube with 1.5 ml tube	
☐ Add 14 µl **DNA Buffer** ○, incubate 1 min at RT, centrifuge 2 min	
☐ Transfer 13 µl of sample to new PCR tube/plate	
☐ Pre-mix 6 µl of **SS** ● and 1 µl of **E3** ● per reaction	2nd Strand Synthesis
☐ Add 7 µl of **SS / E3** mix to each reaction	
☐ Incubate: 98 °C / 90 sec, 62 °C / 60 sec, 72°C / 5 min, 10 °C hold	
☐ Add 160 µl of **CB2** ○, mix and apply to column, centrifuge 1 min	Purification
☐☐ Add 200 µl of **CW** ○, centrifuge 1 min, repeat once	
☐ Discard flow-through, centrifuge 2 min	
☐ Exchange Collection Tube with 1.5 ml tube	
☐ Add 19 µl **DNA Buffer** ○, incubate 1 min at RT, centrifuge 2 min	

300 min Full-Length cDNA Amplification

- [ ] Pre-mix 5 µl of **PCR** ●, 2 µl of **FP** ●, 2 µl of **RP** ●, 1 µl of **E3** ●, 1 µl of SYBR Green I 1:5000 dilution
- [ ] Add 11 µl of **PCR/PFP/PRP/E3/** SYBR Green I (1:5000) mix with up to 9 µl ds cDNA sample
- [ ] qPCR: 98 °C for 30 sec
  50 °C for 90 sec
  72 °C for 5 min
  39 x { 98 °C for 30 sec; 62 °C for 60 sec; 72 °C for 5 min }
  72 °C for 5 min; 10 °C hold

  qPCR
- [ ] Take the cycle number where fluorescence is at 80 % from the maximum for endpoint PCR

- [ ] Pre-mix 5 µl of **PCR** ●, 2 µl of **FP** ●, 2 µl of **RP** ●, 1 µl of **E3** ●, and 1 µl H_2O or **DNA Buffer** ○
- [ ] Add 11 µl of **PCR / PFP / PRP / E3** mix with up to 9 µl ds cDNA sample
- [ ] Endpoint PCR: 98 °C for 30 sec
  50 °C for 90 sec
  72 °C for 5 min
  xx (see p.20) { 98 °C for 30 sec; 62 °C for 60 sec; 72 °C for 5 min }
  72 °C for 5 min; 10 °C hold

  Endpoint PCR

- [ ] Add 160 µl of **CB2** ○, mix and apply to column, centrifuge 1 min
- [ ] Add 200 µl of **CW** ○ centrifuge 1 min, repeat once
- [ ] Discard flow-through, centrifuge 2 min
- [ ] Exchange Collection Tube with 1.5 ml tube
- [ ] Add 21 µl **DNA Buffer** ○, incubate 1 min at RT, centrifuge 2 min

  Purification

7. Appendix A: RNA Requirements

RNA Integrity

Full-length cDNA synthesis relies on high quality input RNA with intact 3' and 5' ends. With low quality RNA some transcripts will not have a poly(A) tail and/or a cap anymore and hence full-length cDNA synthesis is not possible. However, using TeloPrime which tags the poly(A) tail and the cap, preferably full-length mRNAs will be amplified.

The integrity of an RNA sample can be assessed with a variety of methods. We recommend the use of a microfluidics assay such as the RNA6000 series for the 2100 Bioanalyzer (Agilent Technologies Inc.), although RNA quality can also be assessed with denaturing agarose gel electrophoresis if such a device is not available. Most microfluidics platforms will carry out an automated peak analysis and generate a quality score (RIN or RQN), in addition to the 28S / 18S rRNA ratio.

RNA Amount

The amount of total RNA required depends on the poly(A) RNA content and the integrity of the sample in question. This protocol was extensively tested with total RNA extracted from mouse liver, brain, spleen, thymus, kidney, lung, heart, as well as Universal Human Reference RNA (UHR), Human Brain Reference RNA (HBR), and human cell lines. Input amounts of 1 ng – 2 µg of total RNA were successfully used with TeloPrime (see Figure 3 and corresponding Table).

The input requirements for your particular experiment may be different. However, to maintain the quantitative and complex representation of the transcriptome and to avoid the loss of longer cDNAs, it is essential not to overcycle your endpoint PCR. Therefore the protocol requires a qPCR assay to be carried out in order to determine the optimal number of cycles for your endpoint PCR.

Total RNA Input	PCR cycles
2000 ng	16
1000 ng	17
500 ng	18
100 ng	19
50 ng	21
10 ng	23
5 ng	25
1 ng	28

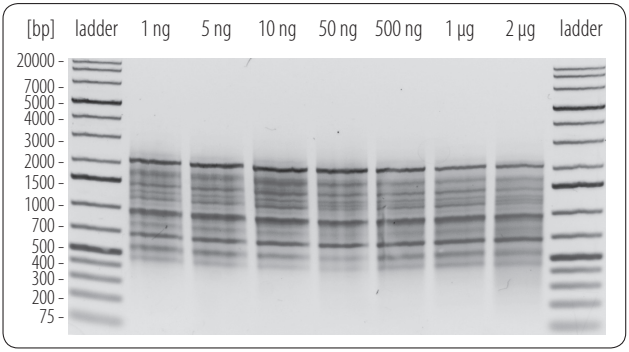


Figure 3. Agarose gel (0.7 %) picture of TeloPrime Full-Length cDNA synthesized from 1 ng to 2 µg total RNA input isolated from Mm Liver. Banding pattern and length is consitent despite different input RNA amounts. The cycle numbers for the endpoint PCRs (shown in the table on the left) were determined as described in Appendix B, p.20.

Potential Contaminants

RNA samples should be free of salts, metal ions, and organic solvents, which can be carried over from the RNA extraction. Several sources of contamination can be detected with an UV-Vis spectrophotometer. An acceptably pure RNA sample should have an A260 / A280 ratio between 1.8 and 2.1. The A260 / A230 ratio should also be approximately 2. Several common contaminants including proteins, chaotropic salts and phenol absorb strongly between 220 and 230 nm and can often be identified as peaks in this region. Contamination with any of these generates a lower A260 / 230 ratio. Phenol also has an absorption maximum between 250 and 280 nm, which overlaps that of nucleic acid, so high 230 nm absorbance combined with a biphasic or broad peak between 250 and 280 nm may indicate contamination with phenol rather than chaotropic salts.

Genomic DNA Contamination

Depending on the RNA extraction protocol used, samples may also contain significant amounts of gDNA, which is indistinguishable from RNA on a spectrophotometer. Furthermore, as many of the dyes used in RNA microfluidics assays stain single-stranded nucleic acids much more intensely than double-stranded, low to moderate amounts of gDNA may not be readily visible with an RNA-specific microfluidics assay. We highly recommend examining all RNA samples on a denaturing agarose gel or using a fluorometric assay with DNA- and RNA-specific dyes to check samples for DNA contamination. On an agarose gel, gDNA can appear as either a dark mass which remains in the slot if relatively intact or as a high molecular weight smear if it has been sheared during extraction.

The best way to avoid gDNA contamination is to use an RNA extraction protocol that does not co-isolate gDNA such as Lexogen's SPLIT RNA Extraction Kit (Cat. No. 008.48). However, DNA can be removed from irreplaceable samples by acidic phenol extraction or DNase I digestion. We do not recommend DNase treatment as the extended incubation with divalent cations can lead to RNA hydrolysis and decrease RNA integrity. If samples must be DNase treated, heat inactivation should be avoided and the enzyme deactivated by other means such as phenol/chloroform extraction or silica column purification.

8. Appendix B: Calculation of the Endpoint PCR

The endpoint PCRs are performed to generate enough material for any subsequent application such as NGS sample preps, microarrays, or cDNA cloning.

The number of cycles for your endpoint PCR depends on the quality of your RNA, the total RNA input, and the mRNA content in RNA extractions, which varies between cell types, tissues, and organisms. To take care of these variables and prevent any over- or undercycling of your samples, which could have negative effects on downstream applications, it is recommended to always carry out a qPCR assay by adding SYBR Green I to the PCR Mix (make sure to use SYBR Green I in a final concentration of 0.1 x). This initial qPCR should be amplified in 40 cycles and evaluated to determine the fluorescence value at which the fluorescence reaches a plateau. Calculate where the fluorescence is at 80 % from the maximum; this is the cycle number to be used for the endpoint PCR with the remaining sample.

NOTE: The PCR program for amplification is a two step PCR with the initial cycle using a lower annealing temperature. The resulting endpoint cycle numbers from the calculation however also includes this cycle. If the cycle number you determined is e.g., 19 this means 1 cycle at the low annealing temperature plus 18 cycles at the higher annealing temperature.

Once this number is established for a certain kind of sample, the same number can be used for further experiments.

9. Appendix C: Full-Length cDNA Amplification Quality Control

Quality control of the amplified full-length cDNA is highly recommended and can be carried out with various methods depending on the available equipment. A thorough quality control procedure should include the analysis of concentration, size distribution, and banding pattern of the amplified products.

Quality Control Methods

The concentration of the PCR products can be measured with an UV-Vis spectrophotometer. Visual control of the banding pattern and the size distribution as well as detection of side-products can be done by analyzing a small volume of sample with microcapillary electrophoresis. Several electrophoresis platforms are available from various manufacturers. For low-to medium-throughput applications, we recommend the Bioanalyzer 2100 with the Agilent DNA 12000 kit (Agilent Technologies, Inc.). Typically 1 ng of the amplified sample is sufficient for analysis. Very basic quality control can also be performed by separating about 100 ng of sample on a 0.7 % agarose gel. Examples of typical banding patterns of TeloPrime Full-Length cDNA Amplifications generated from 500 ng total RNA input from different tissues is depicted in Figure 4. The cycle numbers for the endpoint PCRs were determined as described in Appendix B, p.20.

500 ng Input RNA from	PCR cycles
HBR	19
UHR	16
Mm Liver	18
Mm Kidney	18
Mm Brain	19
Mm Spleen	21
Mm Thymus	19
Mm Lung	22
Mm Heart	22

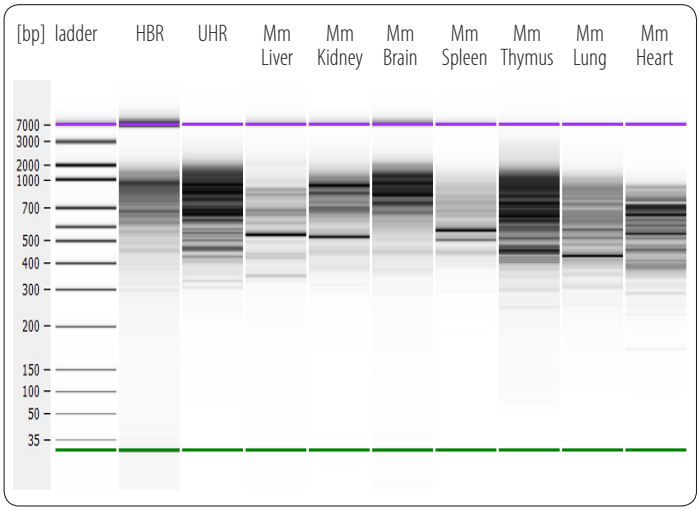


Figure 4. Bioanalyzer gel traces of TeloPrime Full-Length cDNA synthesized from 500 ng total RNA input isolated from Human Brain Reference RNA (HBR), Universal Human Reference RNA (UHR), Mm Liver, Mm Kidney Mm Brain, Mm Spleen, Mm Thymus, Mm Lung, and Mm Heart. The cycle numbers for the endpoint PCRs (shown in the table on the left) were determined as described in Appendix B, p.20.

10. Appendix D: Downstream Applications

The full-length cDNA products can be used for various downstream applications such as Next Generation Sequencing (NGS), RACE, cloning, microarray probes, and normalization.

For further full-length or gene-specific PCRs, Lexogen offers a TeloPrime PCR Add-on Kit (Cat. No.18.30) containing 30 rxn. This kit contains the PCR Forward and Reverse Primer separately, hence they can be substituted with a gene-specific primer of interest.

The PCR primers included in the basic TeloPrime Kit (Cat.No.13.04, 13.08, and 13.24) are of the following sequence:

```
FP: 5′ – TGGATTGATATGTAATACGACTCACTATAG – 3‘
RP: 5′ – TCTCAGGCGTTTTTTTTTTTTTTTTT – 3‘
```

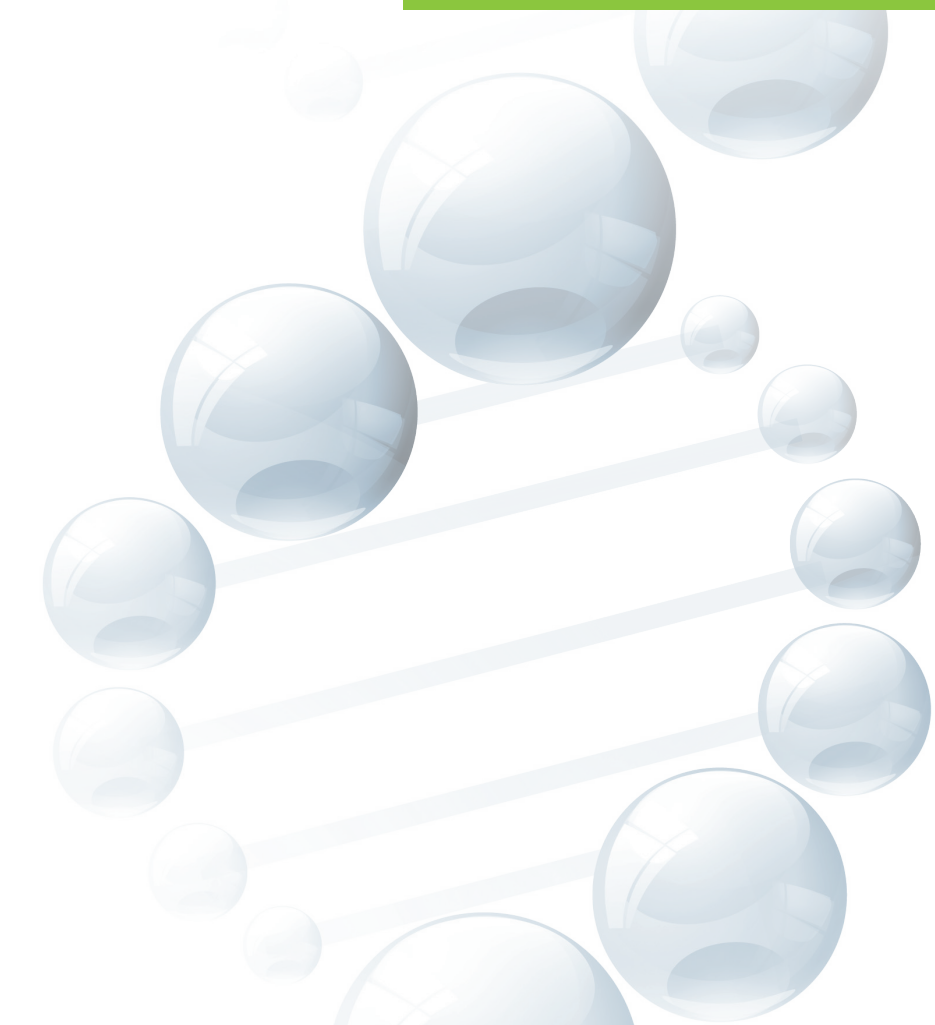
The PCR primers are provided as 5 µM stocks and should be at a final concentration of 0.5 µM in the PCR reaction.
If other primers are used calculate your Tm at a final concentration of 2 mM $MgCl_2$.

11. Appendix E: Revision History

Revision date	Change	Page
September 01st 2014	Initial Release	

LEXOGEN

Enabling complete transcriptome sequencing



TeloPrime Full-Length cDNA Amplification Kit · User Guide

Lexogen GmbH
Campus Vienna Biocenter 5
1030 Vienna, Austria
Telephone: +43 (0) 1 345 1212-41
Fax: +43 (0) 1 345 1212-99
E-mail: info@lexogen.com
© Lexogen GmbH, 2014