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ExactSTART™ Full-Length cDNA Library Cloning Kit

Cat. No. ES0907 – 10 Reactions

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1. Introduction

The ExactSTART Platform of Transcriptome Discovery and Analysis Tools

The ExactSTART Full-Length cDNA Library Cloning Kit is one component of the ExactSTART Platform* of transcriptome discovery and analysis tools from Epicentre. The ExactSTART (START: Selective Tagging and Amplification of RNA Transcripts) Platform kits combine the advantages of T4 RNA Ligase-mediated RNA tagging with a select group of RNA modifying enzymes that have strict enzymatic specificity. When used in a precise order, these enzymes enable the researcher to selectively "tag" the 5′ and 3′ ends of a specific class of RNA, such as 5′-capped RNAs (e.g., eukaryotic mRNAs and eukaryotic viral RNAs), 5′-triphosphorylated RNAs (e.g., some noncoding RNAs), 5′-monophosphorylated RNAs (e.g., miRNAs), as well as other identified RNAs or RNAs whose functions are not yet identified. By incorporating 5′ and 3′ tags with unique functional sequences, the selected class of transcripts can be amplified for a specific downstream application such as next-gen sequencing, RACE, cloning, RT-PCR, microarray, and other gene discovery and analysis methods.

Visit www.Epicentre.com/exactstart to learn more about the ExactSTART Platform and about the currently available ExactSTART Kits.

Features and Benefits of the ExactSTART Full-Length cDNA Library Cloning Kit

1) Full-length cDNA libraries can be produced from as little as 1 µg of total RNA.
2) Extremely strong selection for only full-length cDNAs to appear in the final cloned library.
3) Preservation of the extreme 5′ end of the mRNA molecule allows for the accurate mapping of transcriptional start sites.
4) Cloning of novel non-coding transcripts.
5) Discovery of new, alternate transcription start sites.
6) Unlike other commonly used full-length cDNA synthesis methods, the ExactSTART approach does not add template independent nucleotides to the RNA during the tagging process. The ExactSTART Kit captures the true transcriptional start site, without the sequence ambiguity often created by other systems.
7) Pre-cut, cloning-ready pCDC1-K vector directionally clones tagged cDNAs into rare *Asc* I and *Not* I restriction sites.
8) Streamlined protocol allows non-size-selected libraries to be created and cloned in as little as one day.

The ExactSTART Full-Length cDNA Library Cloning Kit facilitates the precise identification of the 5′- and 3′-ends of both coding and non-coding RNAs. It complements functional genomic studies using DNA microarrays and SAGE (serial analysis of gene expression). These techniques are able to investigate large numbers of genes and identify all the transcripts present in a sample, but do not allow for the identification and annotation of the exact 5′ and 3′ ends of the transcripts. Mapping the 3′ end is relatively easy using traditional cDNA synthesis methods, but the precise and global identification of the 5′ end of a transcript remains technically demanding.

The ExactSTART Kit solves this problem by producing full-length cDNA derived from polyadenylated RNAs that are either capped or phosphorylated at the 5′ end. These RNAs include traditional capped eukaryotic messenger RNAs, mitochondrial RNAs such as the 5′-phosphate-containing human cytochrome oxidase subunit 2 and NAD dehydrogenase subunit 4 and potentially novel coding and non-coding RNAs such as those being defined by the ENCODE project.

Two strategies are incorporated in the cDNA synthesis method used in the ExactSTART Kit to ensure that the cloned transcripts are derived only from full-length RNA.

1. The cap structure or the 5′-phosphate(s) of the RNA is replaced with a RNA oligo adapter that serves as a common priming sequence. This oligo adapter ligation event prevents most RNA degradation products from having the necessary 5′ tag sequence.
2. Oligo-dT priming of first-strand cDNA synthesis selects for RNA molecules that have the characteristic poly(A) tail of eukaryotic mRNA, mitochondrial RNA (human) and perhaps other novel coding and non coding RNAs.

The ExactSTART Full-Length cDNA Library Cloning Kit contains most of the reagents needed for the creation of a directionally cloned cDNA library from as little as 1 µg of total RNA or up to 250 ng of poly(A) RNA. The researcher will need to supply a PCR system, two restriction enzymes and competent *E. coli* cells to complete the cloned library.

2. Kit Contents

The kit components are supplied in tubes with colored caps for easier identification.

Component Name	Tube Label	10 Rxns	Cap Color
ExactSTART 10X TAP Buffer	ExactSTART 10X TAP Buffer	10 µl	Red
Tobacco Acid Pyrophosphatase (TAP)	TAP Enzyme	10 µl	
RiboGuard RNase Inhibitor	RiboGuard RNase Inhibitor	5 µl	
ExactSTART 10X Ligase and RT Buffer	ExactSTART 10X Ligase and RT Buffer	40 µl	
ExactSTART 20X TAP Stop Buffer	ExactSTART 20X TAP Stop Buffer	10 µl	
ExactSTART RNA Acceptor Oligo* (5′-rGAGCGGCCGCCUGCAGGAAA-3′)	ExactSTART RNA Acceptor Oligo	10 µl	
2 mM ATP Solution	2 mM ATP	10 µl	
T4 RNA Ligase	T4 RNA Ligase	10 µl	
ExactSTART 1st-Strand Primer	1st-Strand Primer	10 µl	Yellow
10 mM each dNTPs Solution	10 mM each dNTPs	20 µl	
MMLV-RT	MMLV-Reserve Transcriptase	10 µl	
ExactSTART RNase Mix	ExactSTART RNase Mix	10 µl	
ExactSTART PCR Primer 1	ExactSTART PCR Primer 1	15 µl	Blue
ExactSTART PCR Primer 2	ExactSTART PCR Primer 2	10 µl	
FailSafe PCR 2X PreMix E	FailSafe PCR 2X PreMix E	400 µl	
Fast-Link 10X Ligase Buffer	Fast-Link 10X Ligase Buffer	20 µl	Green
Fast-Link DNA Ligase	Fast-Link Ligase	10 µl	
10 mM ATP Solution	10 mM ATP	20 µl	
pCDC1-K Cloning Vector (20 ng/µl)	pCDC1-K Cloning Vector (20 ng/µl)	10 µl	
DNA Fragment 2X Precipitation Solution	DNA Fragment 2X Precipitation Solution	1.0 ml	Clear
ExactSTART RNA Control Transcript (100 ng/µl)*	ExactSTART RNA Control Transcript	10 µl	
Nuclease-Free Water	Nuclease-Free Water	1.5 ml	

Important! *The components of the ExactSTART Full-Length cDNA Library Cloning Kit are formulated specifically and solely for use with this kit. DO NOT use any component of this kit with any other ExactSTART Kit. DO NOT use any component of another ExactSTART Kit with this kit.*

The researcher will need to supply a PCR system, two restriction enzymes and competent *E. coli* cells to complete the cloned library.

***Storage:** Upon receipt of this kit, immediately remove the tubes containing the ExactSTART RNA Control Transcript and ExactSTART RNA Acceptor Oligo and store them at –70°C. Store the remainder of the kit at –20°C.

Additional Required Reagents and Equipment

Thermostable DNA polymerase (preferably with proofreading activity)

Thermocycler or heating block

Thin-walled PCR tubes

TE Buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA), RNase-free

1:1 phenol (buffered):chloroform

24:1 chloroform:isoamyl alcohol

SOC or similar transformation recovery media

LB/Kanamycin Agar (50 µg/ml)

100% Ethanol (cold)

70% Ethanol (cold)

3 M Sodium acetate

Competent *E. coli* host strain

Asc I restriction enzyme (10 U/µl)

Not I restriction enzyme (10 U/µl)

Performance Specifications and Quality Control

The ExactSTART Full-Length cDNA Library Cloning Kit is function-tested in a control reaction. The kit must produce >10^7 colonies from 250 ng of the ExactSTART RNA Control Template when using Epicentre's TransforMax™ EC100™ Electrocompetent *E. coli* cells.

All components of the ExactSTART Full-Length cDNA Library Cloning Kit are free of detectable RNase and DNase activities as judged by agarose gel electrophoresis following over-digestion assays, with the exception of the inherent nucleolytic functions of the MMLV-RT and ExactSTART RNase Mix components.

3. Kit Overview

The basic workflow of the kit is outlined in Fig. 1. The ExactSTART kit contains the Tobacco Acid Pyrophosphatase (TAP) enzyme, which has the unique ability to remove the 5′ guanosine cap of mRNA leaving a 5′ monophosphate, the substrate for the ligation of a RNA adapter. RNA molecules that normally possess a 5′ monophosphate are not affected by TAP. The RNA adapter oligo is attached to the 5′ monophosphate RNA by T4 RNA Ligase. This RNA adapter contains a tagging sequence that provides a common priming site for the subsequent PCR amplification steps and a restriction site for cloning into the provided vector.

The reverse transcription step uses an oligo(dT) priming strategy that also adds a tag to the 5′ end of the cDNA. This tag sequence provides an efficient PCR priming site for the subsequent PCR amplification steps and also includes a restriction site for cloning the double-stranded DNA product.

PCR amplification is accomplished by using primers that anneal to the two tags added in the previous steps. The ExactSTART Kit allows the researcher to supply the PCR enzymes and buffers that are most suited to their research goals. Epicentre researchers typically use the FailSafe™ PCR System with Buffer E for reliable, high-fidelity amplification of the cDNA library (see Note 10).

Cloning of the PCR-amplified cDNA involves the pCDC1-K vector, which is a high copy-number, pUC-based plasmid with *Not* I and *Asc* I restriction sites ("rare cutters") in its multiple cloning site. pCDC1-K also contains a kanamycin resistance gene that serves as a marker for plasmid selection. The plasmid vector is supplied as pre-cut, cloning-ready DNA to ensure maximum cloning efficiency.

The number of full-length cDNA clones obtained from a sample will vary greatly depending on the RNA source and quality. Since the ExactSTART Kit provides a strong selection for intact, full-length RNA, samples exhibiting degradation may produce significantly fewer clones.

Notes and Considerations

1. **Thin-walled PCR Tubes and Thermocycler:** Researchers are strongly encouraged to use a thermocycler and thin-walled PCR tubes for all of the steps and incubations in this protocol, even those not involving PCR. If a thermocycler is not readily available for all the steps, standard heat blocks can be used, but care should be taken to ensure the temperatures are accurate and excessive sample evaporation does not occur.
2. **Input RNA Considerations:** The quality of the input RNA is critical for both the yield and the representation of the cDNA library. The cDNA synthesis process used in the ExactSTART kit is designed to specifically exclude partially degraded mRNAs that do not have a 5′ phosphate; therefore, the starting RNA material needs to be of extremely high quality. Epicentre offers a broad range of RNA purification kits that are able to purify intact RNA from a wide variety of starting materials.

   RNA should be dissolved in water or standard TE (10 mM Tris-HCl [pH 7.5], 1 mM EDTA).
3. **Standard RNA "Good Practices":** The use of standard laboratory "good practices" is critical when working with RNA due to the ubiquitous presence of RNases on most laboratory surfaces and human skin. The use of gloves, aerosol barrier pipette tips, and certified RNase-Free sample tubes is strongly encouraged. It is also advisable to create an RNase-free area in which work is performed. Surface cleaners that specifically remove and inactivate RNases are available from many vendors.
4. **Stopping Points:** While the cDNA synthesis procedure is streamlined and designed to be performed in less than 1 day, there are a few stopping points indicated in the protocol. We suggest that the protocol be performed all at once, but these spots are designed to provide breaks if needed.
5. **PCR Bias:** There is a chance that the amplification of double-stranded cDNA by PCR might affect the accurate representation of transcript levels in the library. In general, the fewer PCR cycles performed, the smaller these biases will be. For researchers not concerned about the relative expression levels of the cDNAs within the library, higher numbers of PCR cycles are suggested to maximize the number of clones produced.
6. **Incomplete, Non-Full-Length Clones:** The ExactSTART Kit is designed to use two rare, eight-base-cutting restriction enzymes, *Asc* I and *Not* I, which are extremely infrequent cutters of most eukaryotic cDNAs. However, it is possible that certain, specific cDNAs will not be in the final library or only be present in a truncated form, due to the presence of one of these restriction sites inside the specific cDNA. One solution for this situation is cutting the cDNA at the alternative rare, eight-base-cutting restriction sites built-in with the oligonucleotides provided in the ExactSTART Kit; there is an *Sbf* I site adjacent to the *Not* I site produced by the RNA oligo acceptor ligation event, and an *Sgr*A I site located adjacent to the *Asc* I site created by the oligo(dT) primer during cDNA synthesis (see Fig. 2).
   Vectors with the appropriate combination of restriction sites would then need to be provided by the researcher, and are not a component of this kit.

   Another strategy for creating a full-length clone from an incomplete cDNA is to use a RACE (rapid amplification of cDNA ends) technique to generate the missing end. This

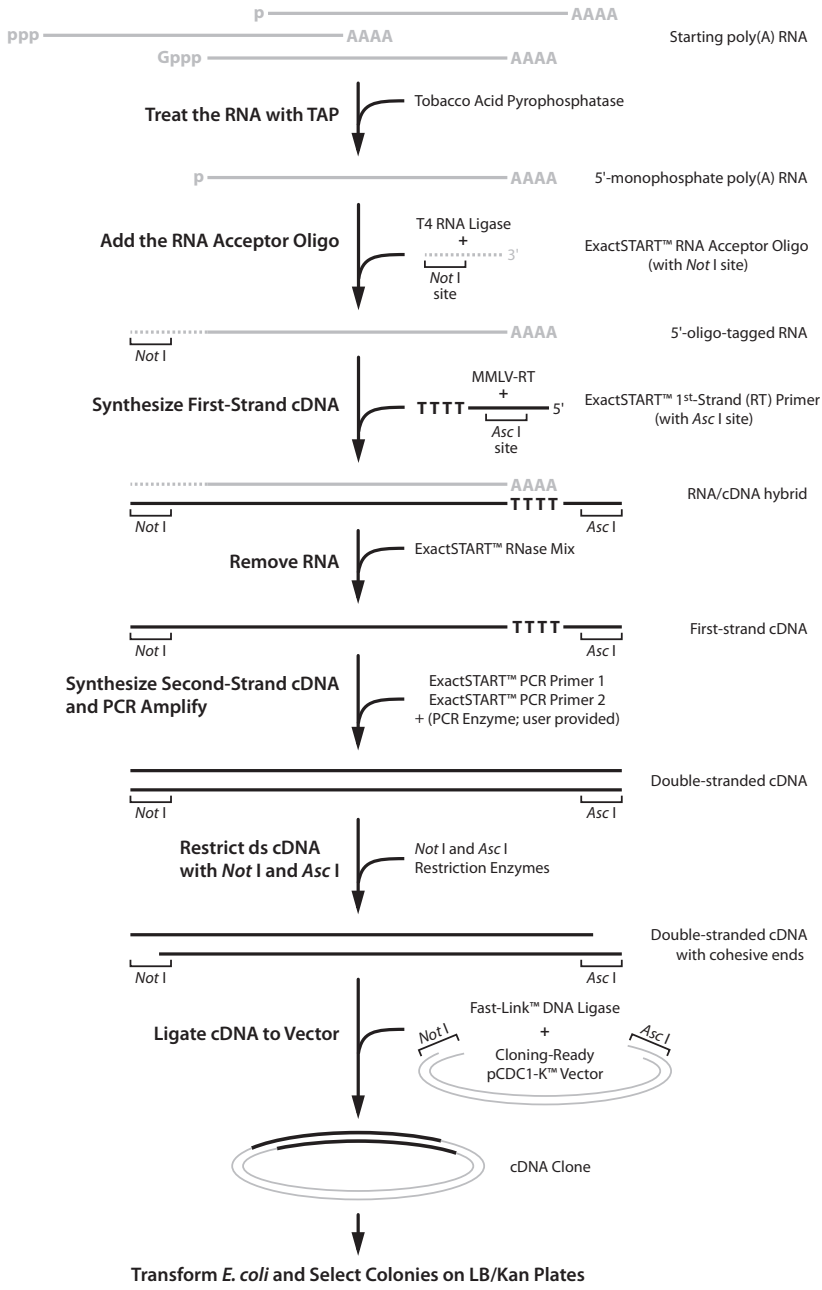


Figure 1. **The ExactSTART™ Full-Length cDNA Library Cloning Kit Procedure.**

technique involves the PCR amplification using the appropriate 5′ or 3′ ExactSTART PCR primer and a gene-specific primer derived from the end of the existing cDNA clone.

7. **Low Yields of PCR-Amplified cDNA:** Low yields of cDNA are likely the result of poor quality (i.e., not full-length) starting RNA. While additional rounds of PCR may increase the yield of cDNA, the representation of the individual cDNAs in the library may be compromised, and repeating the cDNA synthesis process with a different starting RNA purification method may provide better results. See Note 2 for more information regarding purification of intact RNA.
8. **DNA Precipitation:** The ExactSTART Kit includes Epicentre's DNA Fragment 2X Precipitation Solution which is suitable for precipitating DNA fragments over 200 bp in size. If smaller cDNA molecules are to be cloned, alternate purification approaches will be needed.
9. **Size Fractionation:** To obtain better representation of the larger cDNA products, it may be desirable to perform a size fractionation on the PCR-amplified cDNA products prior to cloning. The simplest method for fractionating the cDNA is to use an agarose gel and manually cut out bands of the appropriate size. Care should be taken not to expose the DNA to UV light and ethidium bromide, as this can significantly lower cloning efficiency. A protocol for performing this procedure is included in Appendix B.
10. **PCR Reagents:** The ExactSTART Kit requires PCR amplification of the double-stranded cDNA prior to cloning. We strongly suggest using a proof-reading enzyme such as Epicentre's FailSafe PCR System with PreMix Buffer E for this step, as it will help reduce mutations inherent in PCR amplification. FailSafe PreMix Buffer E is included with the kit. The user will need supply their own thermostable DNA polymerase. If unusual PCR conditions are expected (e.g., high G/C-content cDNAs or extremely long cDNAs) the FailSafe PreMix Selection Kit can be used to optimize the PCR conditions.
11. **Considerations for the use of other PCR Systems:** PCR systems other than Epicentre's FailSafe PCR System can be used with the ExactSTART Kit. There are three important considerations in setting up this reaction. First is the amount of enzyme used; 2.5 U of enzyme in a 100-µl reaction provides good results when using the FailSafe PCR System. The volumes and enzyme amounts may need to be altered if another supplier's enzyme is used.

    Second, the salt and Mg^{2+} concentrations need to be correct for the PCR system used. We suggest performing an additional DNA precipitation step be performed so that the proper conditions can be obtained for the enzyme. This step does not need to be taken if the FailSafe System is used.

    Third, the optimum elongation times for many PCR enzymes, including most proof-reading enzymes, can be much longer than the times outlined in the protocol. Increasing the elongation time may increase DNA yields and better amplify longer sequences.

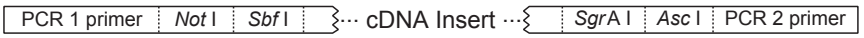


Figure 2. Built-in Restriction Sites.

12. ***E. coli* Cloning Strains:** Any strain of *E. coli* capable of supporting the *ColE*1 plasmid origin of replication (which is used in pUC, pBR, and many other plasmids) can be used to clone and propagate the cDNA library. Epicentre suggests the use of our TransforMax EC100 Electrocompetent or Chemically Competent *E. coli* cells for this purpose. These cells have extremely high transformation competencies and provide a stable, DNA purification-friendly background.

    We have found that cloning libraries in lower copy-number background can often lead to more clones being recovered; this is due primarily to the incidental expression of gene products that are toxic to *E. coli* from the cDNA insert. Epicentre offers CopyCutter™ EPI400™ *E. coli* cells for this added safety (see Related Products).

4. ExactSTART Full-Length cDNA Library Cloning Kit Procedure

Important! *The components of the ExactSTART Full-Length cDNA Library Cloning Kit are formulated specifically and solely for use with this kit. DO NOT use any component of this kit with any other ExactSTART Kit. DO NOT use any component of another ExactSTART Kit with this kit.*

Please review the Procedure (parts A-E) and read all of the Notes and Considerations before attempting the procedure. If you have any questions please contact our Technical Services staff.

A. RNA Decapping and Oligo Ligation

The RNA decapping step uses TAP to remove the 5′-guanosine cap from the mRNA in the total RNA sample. TAP will convert 5′-triphosphate RNA molecules into 5′-monophosphate molecules as well. RNA molecules that have 5′-monophosphates prior to TAP treatment will not be affected by TAP.

T4 RNA Ligase is then used to attach the RNA oligonucleotide to the 5′ end of the mRNA. This oligo serves as a template for the creation of both the PCR priming site and the *Not* I restriction site used for cloning. If removal of *in vivo* mRNA degradation products is required, the alkaline phosphatase pretreatment protocol (Appendix A) can be performed prior to beginning Part A.

The TAP treatment steps may be omitted to exclusively clone RNA molecules with 5′ monophosphates and 3′-poly(A) tails. Human mitochondrial mRNA coding for cytochrome oxidase subunit 2 is an example of this RNA type. It is possible that other coding and non-coding RNAs fall into this category as well.

Required in Part A

Component Name	Tube Label	Cap Color
ExactSTART 10X TAP Buffer	ExactSTART 10X TAP Buffer	Red
RiboGuard RNase Inhibitor	RiboGuard RNase Inhibitor	
Tobacco Acid Pyrophosphatase (TAP)	TAP Enzyme	
ExactSTART 10X Ligase and RT Buffer	ExactSTART 10X Ligase and RT Buffer	
ExactSTART 20X TAP Stop Buffer	ExactSTART 20X TAP Stop Buffer	
ExactSTART RNA Acceptor Oligo	ExactSTART RNA Acceptor Oligo	
2 mM ATP Solution	2 mM ATP	
T4 RNA Ligase	T4 RNA Ligase	
Nuclease-Free Water	Nuclease-Free Water	Clear

1. Assemble the following reagents on ice, in the order given, in a thin-walled PCR tube:

x	µl	Nuclease-Free Water
1	µl	ExactSTART 10X TAP Buffer
0.5	µl	RiboGuard RNase Inhibitor
x	µl	Total RNA sample (1-10 µg, See Note 2)
1	µl	TAP
10	µl	Total reaction volume

2. Incubate the reaction at 37°C for 30 minutes.
3. Remove the tube from 37°C incubation and keep the tube at room temperature.
4. Add the following reagents to the tube.
   Mix each reagent in the tube by gently pipetting several times.
   Be careful to add the reagents in the same order as they are listed here.
   Do NOT use a master-mix tube when processing multiple samples.

   Important: *Be sure to use the 2 mM ATP in the Red-cap tube in this step.*

10	µl	TAP-treated RNA from Part A, Step 1
4	µl	Nuclease-Free Water
2	µl	ExactSTART 10X Ligase and RT Buffer
1	µl	ExactSTART 20X TAP Stop Buffer
1	µl	ExactSTART RNA Acceptor Oligo
1	µl	2 mM ATP Solution
1	µl	T4 RNA Ligase
20	µl	Total reaction volume

5. Incubate the reaction at 37°C for 30 minutes.
6. Remove the tube from 37°C incubation. Proceed immediately to Part B (below).

B. cDNA Synthesis and RNA Removal

This portion of the protocol simultaneously creates the first-strand cDNA and tags it with the necessary amplification and restriction sequences.

Required in Part B

Component Name	Tube Label	Cap Color
ExactSTART 1st-Strand Primer	cDNA Synthesis Primer	Yellow
10 mM each dNTPs Solution	10 mM each dNTP's	
ExactSTART 10X Ligase and RT Buffer	ExactSTART 10X Ligase and RT Buffer	Red
MMLV-RT	MMLV Reverse Transcriptase	Yellow
ExactSTART RNase Mix	ExactSTART RNase Mix	
Nuclease-Free Water	Nuclease-Free Water	Clear

1. Add the following reagents to the reaction tube at room temperature.
   Pipette each reagent several times to mix thoroughly.

20	µl	RNA Acceptor Oligo-ligated RNA from Part A, Step 6 (above)
14	µl	Nuclease-Free Water
1	µl	ExactSTART 1st-Strand Primer
2	µl	10 mM each dNTPs Solution
2	µl	ExactSTART 10X Ligase and RT Buffer
1	µl	MMLV-RT
40	µl	Total reaction volume

2. Incubate the reaction at 37°C for 1 hour.
3. Incubate the reaction at 85°C for 10 minutes to heat-inactivate the MMLV-RT.
   Then incubate the tube at 55°C for the downstream reactions.
4. Add 1 µl of ExactSTART RNase Mix to the reaction and incubate at 55°C for 5 minutes.
   The reaction should be kept at 55°C until the PCR amplification in Part C.

C. Second-Strand cDNA Synthesis and Amplification

The ExactSTART Kit uses a PCR amplification step to create both the second strand of the cDNA and amplify the double-stranded cDNA to amounts that can be efficiently cloned. Epicentre recommends the use of a proof-reading enzyme such as the FailSafe PCR System (see Notes 10 and 11). The PCR enzyme will need to be supplied by the researcher (see Note 10).

The following protocol is designed for use with the FailSafe PCR System. Adjustments may be needed if other PCR systems are used for amplification.

Required in Part C

Component Name	Tube Label	Cap Color
ExactSTART PCR Primer 1	ExactSTART PCR Primer 1	Blue
ExactSTART PCR Primer 2	ExactSTART PCR Primer 2	
FailSafe PCR 2X Premix E	FailSafe PCR Premix E	
DNA Fragment 2X Precipitation Solution	DNA Fragment 2X Precipitation Solution	Clear
Nuclease-Free Water	Nuclease-Free Water	

Additional Required Reagents (provided by the user)

Thermostable DNA polymerase (preferably with proofreading activity)

1:1 Phenol (buffered):chloroform

Chloroform:isoamyl alcohol (24:1)

1. Add the following reagents to the reaction tube at 55°C:

41	µl	RNase-treated first-strand cDNA from Part B, Step 4
26	µl	Nuclease-Free Water
1	µl	ExactSTART PCR Primer 1
1	µl	ExactSTART PCR Primer 2
30	µl	FailSafe PCR Premix E
1	µl	FailSafe PCR Enzyme (2.5 Units)
100	µl	Total reaction volume

2. Cycle the sample in a thermocycler under the following conditions:

   95°C for 30 seconds (initial denaturation)

   followed by 12–18 cycles of: (see Note 5)

   95°C for 30 seconds

   60°C for 30 seconds

   72°C for 4 minutes

   Hold at 4°C.

   This is a convenient stopping point for the reaction process. If desired, the samples can be stored at 4°C for later use.

3. Add 100 µl of 1:1 phenol (buffered) : chloroform to the sample. Vortex vigorously for 15 seconds.
4. Centrifuge the sample for 1 minute at >10,000 x g. Carefully remove the upper, aqueous layer and transfer it to a new tube.
5. Add 100 µl of chloroform:isoamyl alcohol (24:1) to the sample. Vortex vigorously for 15 seconds.

6. Centrifuge the sample for 1 minute at >10,000 x g.
   Carefully remove the upper, aqueous layer and transfer it to a new tube.
   Measure the volume of the aqueous layer.
7. Recover the amplified double-stranded cDNA using the DNA Fragment 2X Precipitation Solution (see Note 8). Add an equal volume of the DNA Fragment 2X Precipitation Solution to the tube and vortex vigorously. Incubate the tube for 10 minutes on ice.
8. Centrifuge the tube at >10,000 x g for 15 minutes at 4°C.

   Note: *It may be necessary to transfer the sample to a thicker-walled tube for this step.*
9. Carefully remove and discard the supernatant. Wash the cDNA pellet by adding 200 µl of 70% ethanol to the tube.
10. Centrifuge the tube at >10,000 x g for 15 minutes at 4°C.
11. Carefully remove and discard the supernatant. Take care not to disturb the pellet. Air dry the cDNA pellet at room temperature for 10 minutes.
12. Resuspend the cDNA pellet in 25 µl of Nuclease-Free Water.
13. Quantitate the amount of cDNA present in the sample by fluorimetry or A_{260} absorbance.

    See Notes 1 and 7 if lower than expected amounts of cDNA are obtained after this step.

This is a convenient stopping point for the reaction process. If desired, the samples can be stored at –20°C for later use.

D. Double-Strand cDNA Restriction Endonuclease Digestion

Before cloning the PCR-amplified cDNA, it may be desirable to perform a size selection on the DNA to obtain a better representation of the cDNA of desired size. Please refer to Appendix B for more information on this optional step.

Required in Part D

Component Name	Tube Label	Cap Color
Nuclease-Free Water	Nuclease-Free Water	Clear

Additional Required Reagents (provided by the user)

Asc I restriction endonuclease (10 U/µl) and reaction buffer

Not I restriction endonuclease (10 U/µl) and reaction buffer

Alternately, *Bss*H II and *Eag* I restriction endonucleases may be used in place of *Asc* I and *Not* I respectively.

Note: *BssH II and Eag I have a six-basepair recognition sequence, compared to Asc I and Not I which have an eight-basepair recognition sequence.*

1. Perform a double restriction digest on the PCR-amplified cDNA from Part C, Step 13 using the restriction enzymes *Not* I and *Asc* I (provided by the user). The supplied pCDC1-K vector has been precut at these sites, and does not need to be digested. A suggested protocol is listed in Part D, Step 2 below; consult the restriction enzyme manufacturer's information for more specific recommendations.

x	µl	Nuclease-Free Water
2	µl	10X Restriction Enzyme Buffer
x	µl	PCR-amplified cDNA (up to 1 µg) from Part C, Step 13
1	µl	*Asc* I Restriction Enzyme (10 U/µl)
1	µl	*Not* I Restriction Enzyme (10 U/µl)
20	µl	Total reaction volume

2. Incubate the restriction digest at 37°C for 2 hours.

   Heat-inactivate the restriction enzymes by incubating at 70°C for 20 minutes.

E. Cloning of Restriction Endonuclease-Digested Double-Strand cDNA

The ExactSTART Full-Length cDNA Library Cloning Kit contains the Cloning-Ready pCDC1-K Cloning Vector which has been precut at its *Not* I and *Asc* I restriction sites and dephosphorylated. A map of the pCDC1-K Vector is presented in Appendix E. See Note 6 for more information regarding alternate cloning strategies.

Required in Part E

Component Name	Tube Label	Cap Color
Fast-Link DNA Ligase	Fast-Link Ligase	Green
Fast-Link 10X Ligase Buffer	Fast-Link 10X Ligase Buffer	
10 mM ATP	10 mM ATP	
pCDC1-K Cloning Vector (20 ng/µl)	pCDC1-K Cloning Vector	

Additional Required Reagents (provided by the user)

Competent *E. coli* cloning strain (see Note 5)

LB-kanamycin (50 µg/ml) agar plates

1. Heat denature the DNA components. Combine up to 6 µl of restricted PCR-amplified cDNA from Part D, Step 2 with 1 µl of pCDC1-K Cloning-Ready Vector (20 ng/µl) and incubate at 65°C for 5 minutes. Allow the mixture to cool to room temperature. Centrifuge briefly to collect the contents to the bottom of the tube.

   Note: *Use a 2-5 fold molar ratio excess of insert to vector.*

2. Ligate the cDNA and pCDC1-K Vector. Assemble the following reagents:

   Important! *Be sure to use the 10 mM ATP in the Green-cap tube in this step.*

7	µl	heat-denatured from Part E, Step 1
1	µl	Fast-Link 10X Ligase Buffer
1	µl	10 mM ATP Solution
1	µl	Fast-Link DNA Ligase
10	µl	Total reaction volume

3. Incubate the ligation mixture at room temperature (18–22°C) for 10 minutes. Heat-inactivate the ligase by incubating at 65°C for 10 minutes.
4. Using 1 µl of the ligation mixture, transform competent *E. coli* cells (provided by the user).

   Electroporation is the preferred transformation method, as this will typically provide more clones than chemical transformation. High-efficiency TransforMax EC100 Electrocompetent *E. coli* cells from Epicentre are ideal for this application. See Related Products.
5. Rescue the cells in media appropriate for the transformation method used. Incubate the cells at 37°C for 45–60 minutes to allow for cell recovery and expression of the kanamycin resistance gene.
6. Plate the cells on LB-kanamycin (50 µg/ml) agar and incubate overnight at 37°C. Plating several dilutions of the transformation mixture will be necessary to obtain individual colonies.

5. Appendix

Appendix A: Optional Alkaline Phosphatase Pretreatment of Total RNA

This procedure will remove the 5′-monophosphate from RNA transcripts in the total RNA. This is typically done to ensure that ribosomal RNAs do not constitute a significant portion of the amplified cDNA. This treatment will, however, prevent some true mRNA transcripts which are naturally not capped from being amplified, as well. Epicentre researchers have found that treatment with alkaline phosphatase is not necessary in most circumstances, and this step is not suggested if cloning novel RNA transcripts is desired.

When performing this step, we recommend the use of Epicentre's APex™ Heat-Labile Alkaline Phosphatase due to its high activity and purity. Other alkaline phosphatases will work, but it is absolutely critical to remove all phosphatase activity before proceeding to the next step. The protocol below recommends clean up via phenol:chloroform extraction before the RNA is used in the ExactSTART protocol. Alternative means of RNA clean up will also work. See the manufacturer's instructions for alternative clean up procedures. In all cases, heat inactivation of the alkaline phosphatase should be avoided as it will cause degradation of the RNA sample due to the Mg^{2+} present in most phosphatase buffers.

1. Assemble the following reaction:

x	µl	Nuclease-Free water
10	µl	APex 10X Reaction Buffer
x	µl	Total RNA (1 – 10 µg)
5	µl	APex Heat-Labile Alkaline Phosphatase
100	µl	Total reaction volume

2. Incubate the reaction at 37°C for 15 minutes.
3. Add 100 µl of 1:1 phenol (buffered) : chloroform to the sample. Vortex vigorously for 15 seconds.
4. Centrifuge the sample for 1 minute at >10,000 x g. Carefully remove the upper, aqueous layer and transfer it to a new tube.
5. Add 100 µl of chloroform:isoamyl alcohol (24:1) to the sample. Vortex vigorously for 15 seconds.
6. Centrifuge the sample for 1 minute at >10,000 x g. Carefully remove the upper, aqueous layer and transfer it to a new tube.
7. Add 10 µl of 3 M sodium acetate and 300 µl of 100% ethanol. Vortex vigorously. Incubate the tube on ice for 30 minutes.
8. Centrifuge the tube at >10,000 x g for 15 minutes at 4°C.
9. Carefully remove and discard the supernatant. Wash the RNA pellet by adding 200 µl of 70% ethanol to the tube.
10. Centrifuge the tube at >10,000 x g for 15 minutes at 4°C.
11. Carefully remove and discard the supernatant. Take care not to disturb the pellet. Air dry the RNA pellet at room temperature for 10 minutes.
12. Resuspend the RNA pellet in Nuclease-Free Water or TE Buffer (pH 7.5) to a minimum concentration of 1 µg/7.5 µl (see Part A, Step 1).

Appendix B: Size Selection of the cDNA

To maximize the representation of longer cDNAs in the final library, we suggest performing a size selection protocol be performed and the cDNA from the various fractions be cloned individually. Included is a protocol for performing this size selection without exposing the cDNA to UV light and ethidium bromide. Other size selection protocols can be used as well.

Fractionate the DNA by running it on a low-melting-point (LMP) 1% agarose gel. Run the gel for as long as possible to obtain maximal size separation of the cDNA molecules. Use a DNA ladder appropriate for the size of DNA that is to be recovered.

Do not add ethidium bromide to the gel. The DNA that will be cloned should not be exposed to UV light under any circumstances. Even short exposure can decrease the cloning efficiency by 100-fold or more. A diagram of our method is shown in Fig. 3.

Note: *The protocol below is designed for use with Epicentre's GELase™ Agarose Gel-Digestion Preparation, and thus requires LMP agarose. Standard high-melt agarose can also be used and the DNA can be extracted from the gel slices with other chemical-based methods or*

electroelution. Other staining methods which use dyes that do not require exposure to UV light may be used as well.

1. Prepare a 1% LMP agarose gel in 1X TAE or 1X TBE buffer.

   The use of long gels will allow for better resolution.

   Note: *Do not include ethidium bromide in the gel solution.*

2. Load the DNA size marker into both of the outside lanes of the gel and add the cDNA samples to the inner lanes.
3. Resolve the samples by gel electrophoresis at a constant voltage. Typically, running a gel at lower voltages for longer periods of time provides better size resolution.
4. Following electrophoresis, cut off the outer lanes of the gel containing the DNA size marker, (see Fig. 3).
5. Stain the cut-off outer lanes of the gel with ethidium bromide and visualize with UV light.

   Mark the position of the desired size DNA in the gel using a pipette tip.

   Note: *Do not expose the sample DNA to UV irradiation!*

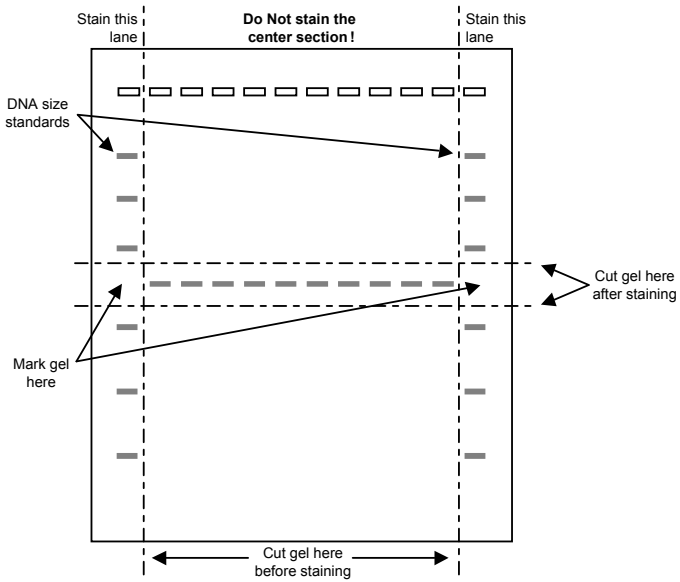


Figure 3. Size-Selected Gel Purification of the cDNA.

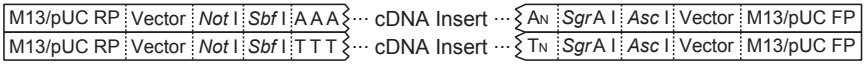


Figure 4. Full-Length cDNA Clone DNA Sequencing Landmarks.

6. Reassemble the gel and excise an appropriately sized gel slice from the inner lanes of the gel containing the sample DNA that migrated with and just slightly above (i.e., higher MW) the marked position of the outer lanes of the gel.
7. Proceed with DNA recovery using the GELase Agarose Gel-Digesting Preparation or other method (e.g., electroelution) for isolating DNA from agarose gels. Follow the protocol appropriate for the recovery method chosen.

Appendix C: Control Reaction for Testing the ExactSTART Full-Length cDNA Library Cloning Kit

The ExactSTART Full-Length cDNA Library Cloning Kit is provided with a control 1.3 kb RNA transcript that can be used to test the performance of the kit.

1. Perform Parts A–D of the protocol using 250 ng (2.5 µl) of the ExactSTART Control RNA Transcript as the starting RNA sample.
2. When plating the transformed cells, use a 1:100 dilution (990 µl of fresh recovery media + 10 µl of the transformed *E. coli*). Plate two plates each with 10 µl and 100 µl of the 1:100 dilution.
3. Incubate the plates at 37°C overnight.
4. Count the colonies and calculate the total number of colonies expected from the full control reaction. The ExactSTART Kit should produce $>10^7$ colonies from the ExactSTART RNA Control Transcript reaction when using electrocompetent *E. coli* cells.
5. If desired, individual colonies can be prepped and the DNA digested with *Not* I and *Asc* I. After electrophoresis and staining, the resulting inserts in the plasmids should all be approximately 1.3 kb in size.

Appendix D: Library DNA Sequencing

The ExactSTART Full-Length cDNA Library Cloning Kit uses the pCDC1-K Cloning Vector which contains M13/pUC Forward and Reverse sequencing primer binding sites located near the multiple cloning site of the vector. The nucleotide sequences for both primers are listed below.

M13/pUC Forward Sequencing Primer:5′- CGCCAGGGTTTTCCCAGTCACGAC-3′

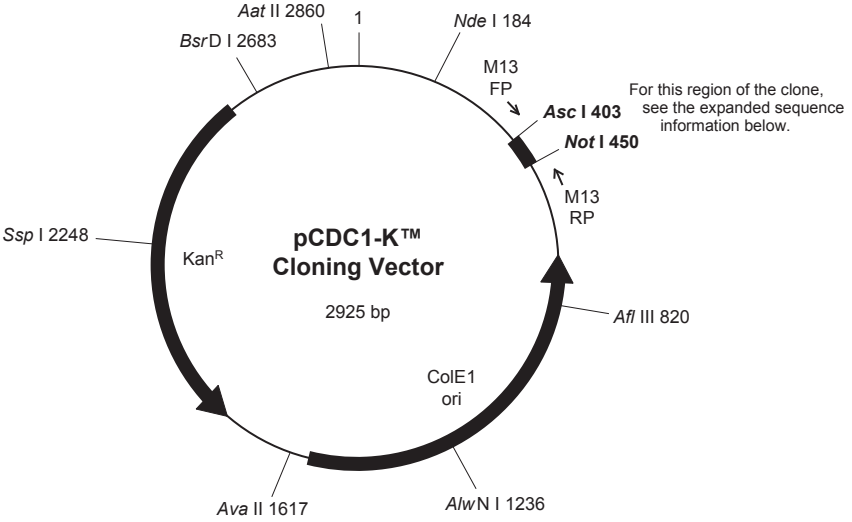
M13/pUC Reverse Sequencing Primer: 5′- AGCGGATAACAATTTCACACAGGA-3′

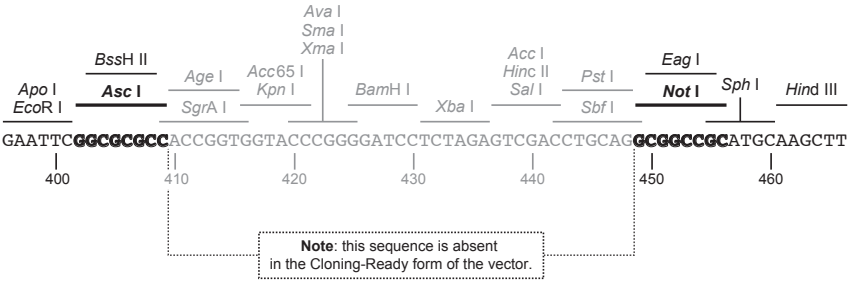
Due to the uniform nature of the 5′ and 3′ tagging sequences found on all of the cDNAs, full-length clones can easily be identified by the series of sequence landmarks unique to these tags. Clones which do not display these landmark patterns are probably not full-length, and could be the result of an internal *Not* I or *Asc* I restriction site. See Note 6 for additional information on the recovery of these sequences.

To determine whether the cDNA does indeed have an internal restriction site, analyze the sequences from both the M13/pUC Forward and Reverse sequencing primers. If the sequence from the M13/pUC Forward primer does not show a poly(A) region after the *Asc* I cut site, then the clone is likely truncated, and a 3′-RACE experiment should be performed to obtain the missing fragment.

If the M13/pUC Reverse primer sequence does not show an *Sbf* I site followed by three A's (CCTCGAGGAAA), then a 5′-RACE experiment should be performed to obtain the missing end of this cDNA.

Appendix E: pCDC1-K™ Vector Information





M13/pUC Forward Sequencing Primer 5' CGCCAGGGTTTTCCCAGTCACGAC 3'
M13/pUC Reverse Sequencing Primer 5' AGCGGATAACAATTTCACACAGGA 3'

Figure 5. pCDC1-K™ Cloning Vector.

The pCDC1-K Cloning Vector 2925 bp. sequence can be downloaded at http://www.epicentre.com/sequences.

Restriction Enzymes that cut the pCDC1-K Cloning Vector one to three times:

Enzyme	Sites	Location	Enzyme	Sites	Location
Aat II	1	2860	Drd I	2	97, 928
Acc65 I *	1	416 *	Dsa I	2	1588, 1649
Acc I *	1	438 *	Eae I *	3	388, 450 *, 659
Afl III	1	820	Eag I	1	450
Age I *	1	410 *	Ear I	3	296, 704, 2357
AlwN I	1	1236	EcoN I	1	2260
ApaB I	1	186	EcoO109 I	1	2914
ApaL I	2	177, 1134	EcoR I	1	396
Apo I	3	396, 2372, 2556	Fsp I	1	258
Asc I	1	403	Hae II	3	239, 698, 1068
Ase I	3	591, 650, 1972	Hinc II *	1	439 *
AsiS I	1	2175	Hind III	1	461
Ava I *	2	420 *, 2571	Hpy99 I	3	376, 389, 925
Ava II	1	1617	Kpn I *	1	420 *
BamH I *	1	425 *	Nar I	1	236
Ban I	3	235, 416, 564	Nde I	1	184
Ban II	1	2522	Not I	1	450
Bau I	1	993	Nru I	1	2516
BciV I	2	1023, 2775	Nsi I	2	2061, 2327
Bfa I *	3	432 *, 1315, 1568	Nsp I	3	41, 459, 824
BfrB I	2	2059, 2325	PaeR7 I	1	2571
BfuA I *	1	450 *	Pci I	1	820
Bgl I	1	251	PflM I	1	1913
Bme1580 I	2	181, 1138	Pfo I	1	46
Bmr I	1	359	PspX I	1	2571
Bpu10 I	1	2153	Pst I *	1	447 *
BsaH I	2	236, 2857	Pvu I	2	279, 2175
BseY I	1	1124	Pvu II	2	308, 644
BsiHKA I	2	181, 1138	Rsa I *	3	169, 418 *, 2338
Bsm I	2	2214, 2291	Sal I *	1	437 *
BsmB I	2	45, 2153	Sap I	1	704
Bsp1286 I	3	181, 1138, 2522	Sau96 I	3	286, 1617, 2914
BspD I	2	2480, 2711	Sbf I *	1	447 *
BspLU11 I	1	820	Sfc I *	3	443 *, 1085, 1276
BspM I *	1	450 *	Sfo I	1	237
BspQ I	1	512	SgrA I *	1	410 *
BsrB I	3	512, 753, 2779	Sim I	2	1012, 1495
BsrD I	1	2683	Sma I *	1	422 *
BsrF I *	2	410 *, 2215	Sph I	1	459
BssH II	1	403	Ssp I	1	2248
BssS I	2	993, 2909	Tat I	1	167
BstAP I	1	185	Tli I	1	2571
BstDS I	2	1592, 1653	Tsp45 I	3	56, 367, 2021
Btg I	2	1588, 1649	TspM I *	1	420 *
Bts I	3	606, 2227, 2314	Xba I *	1	431 *
BtsC I	2	327, 2544	Xho I	1	2571
Cla I	2	2480, 2711	Xma I *	1	420 *
CviQ I *	3	168, 417 *, 2337	Zra I	1	2858

Note: *Bases 410-448 are absent in the Cloning-Ready form of the vector.**

Restriction Enzymes that cut the pCDC1-K Cloning Vector 4 or more times:

Aci I	BstN I	Hae I	Mae II	Ple I
Alu I	BstU I	Hae III	Mae III	PspG I
Alw I	BstY I	Hha I	Mbo I	Sau3A I
Bcc I	Cac8 I	Hinf I	Mbo II	ScrF I
BsaJ I	Cvi II	HinP I	Mly I	SfaN I
BsaW I	CviJ I	Hpa II	MnI I	SmI I
BsiE I	CviKI-1	Hph I	Mse I	Taq I
Bsl I	Dde I	Hpy188 I	Msp I	Tfi I
BsmA I	Dpn I	Hpy188 III	MspA1 I	Tse I
BspH I	Fat I	HpyAV	Mwo I	Tsp4C I
Bsr I	Fau I	HpyCH4 III	Nci I	Tsp509 I
BssK I	Fnu4H I	HpyCH4 IV	Nla III	TspR I
BstF5 I	Gdi II	HpyCH4 V	Nla IV	

Restriction Enzymes that do not cut the pCDC1-K Cloning Vector:

AcI I	Bmt I	Dra III	Pac I	Sca I
Afe I	Bsa I	Eco47 III	Pas I	SexA I
Afl II	BsaA I	EcoR V	PflF I	Sfi I
Ahd I	BsaB I	Fse I	Pme I	SnaB I
Ale I	BsiW I	Hpa I	PmI I	Spe I
Apa I	BspE I	Mfe I	PpuM I	Srf I
Avr II	BsrG I	Mlu I	PshA I	Sse8647 I
Bbs I	BstB I	Msc I	Psi I	Stu I
BbvC I	BstE II	MsI I	PspOM I	Sty I
Bcl I	BstX I	Nae I	Rsr II	Swa I
Bgl II	BstZ17 I	Nco I	Sac I	Tth111 I
Blp I	Bsu36 I	NgoM IV	Sac II	Xcm I
BmgB I	Dra I	Nhe I	SanD I	Xmn I

6. Kit Components Available Separately

Tobacco Acid Pyrophosphatase (includes 10X Reaction Buffer)

T19050	50 Units
T19100	100 Units
T19250	250 Units
T19500	500 Units

T4 RNA Ligase (includes 10X Reaction Buffer and ATP Solution)

LR5010	1,000 Units
LR5025	2,500 Units
LR5050	5,000 Units

RiboGuard™ RNase Inhibitor

RG90925	2,500 Units

FailSafe™ PCR 2X PreMix E

FSP995E	2.5 ml

Fast-Link™ DNA Ligation Kit (includes 10X Reaction Buffer and ATP Solution)

LK11025	25 Ligations
LK0750H	50 Ligations
LK6201H	100 Ligations

Additional ExactSTART™ Kits

ExactSTART™ Eukaryotic mRNA 5′- & 3′-RACE Kit

ES80910 — 10 Reactions

Facilitates the precise mapping of alternative transcription start sites and polyadenylation sites of eukaryotic mRNAs.

Visit www.epicentre.com/exactstart to learn more about the ExactSTART Platform and about the currently available ExactSTART Kits.

7. Related Products

MasterPure™ RNA Purification Kit

MCR85102 — 100 Purifications

The MasterPure RNA Purification Kit provides all reagents needed to purify total RNA from >10,000 eukaryotic cells. The kit is compatible with a wide variety of cell and tissue types.

MasterPure™ Yeast RNA Purification Kit

MPY03010	10 Purifications
MPY03100	100 Purifications

The MasterPure Yeast RNA Purification Kit provides all reagents needed to purify RNA from yeast cells without the use of organic solvents, such as phenol, or columns.

APex™ Heat-Labile Alkaline Phosphatase

AP49010	10 Reactions
AP49050	50 Reactions
AP49100	100 Reactions

APex Phosphatase is a highly active, broad range phosphatase that removes phosphates from all types of DNA and RNA ends. It comes from a recombinant source and does not contain the impurities often encountered in other types of alkaline phosphatases.

GELase™ Agarose Gel-Digesting Preparation

G09050	50 Units
G09100	100 Units
G09200	200 Units

The GELase Preparation is ideal for the recovery of cDNA fragments from LMP agarose gels after size selection.

TransforMax™ EC100™ Electrocompetent and Chemically Competent *E. coli*

EC10005	5 x 100 µl
EC10010	10 x 100 µl
CC02810	10 x 50 µl

TransforMax EC100 Electrocompetent *E. coli* are an ideal choice to recover and propagate the ExactSTART cDNA library. They yield >1 x 10^{10} transformants per microgram of supercoiled DNA.

TransforMax EC100 Chemically Competent cells yield >1 x 10^{8} transformants per microgram of supercoiled DNA.

CopyCutter™ EPI400™ Electrocompetent and Chemically Competent *E. coli* and Induction Solution

C400EL10	10 x 50 µl
C400CH10	10 x 50 µl
CIS40025	25 ml

CopyCutter cells lower the copy number of *ColE*1 high-copy plasmids. Lower copy number can reduce transcriptional activity, allowing many otherwise toxic cDNAs to be cloned. Normal plasmid copy number can be achieved using the CopyCutter Induction Solution.

[+]Use of betaine in DNA polymerase reactions, including, but not limited to use for PCR or DNA sequencing, is covered by U.S. Patent No. 6,270,962, European Patent No. 0742838, German Patent No. DE4411588C1, and other patents or patent applications in the U.S. and other countries that are either assigned or exclusively licensed to Epicentre. Purchase of a product from Epicentre that contains betaine and a thermostable DNA polymerase is accompanied by a limited non-exclusive license for the purchaser to use the purchased product solely for life science research, whether the purchaser performs research in a not-for-profit or a for-profit organization. However, if the product does not contain a thermostable DNA polymerase in addition to betaine, all for-profit organizations require a license from Epicentre in order to use betaine or a product that contains betaine in DNA polymerase reactions for research applications, and not-for-profit organizations require a license if the product, the research, or the result of the research is transferred to or obtained for or on behalf of a for-profit organization. Licenses are also available to use betaine in DNA polymerase reactions for human or animal diagnostics, screening, or other fields of use. Please contact Epicentre for information related to such licenses.

The ExactSTART technology is covered by patents pending.

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