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## Use of EZ::TN™ Transposomes™ for Genetic Analysis and Direct Sequencing of Bacterial Genomic DNA

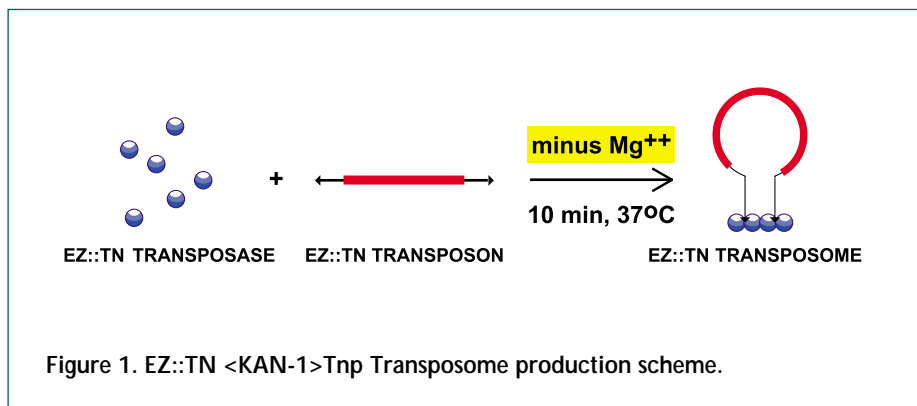
Les Hoffman and Jerry Jendrisak, EPICENTRE Technologies

### Introduction

The genomes of over sixty bacterial species are sequenced or are currently undergoing sequencing. Sequencing tools have now progressed to the point at which the chromosomal DNA of small bacterial genomes can be directly sequenced without molecular cloning.<sup>1</sup> Although direct sequencing has not been attempted for large-scale sequencing projects, the ability to directly sequence bacterial DNA has applications in gap filling and characterization of mutations. In addition, as more bacterial genome data is collected there is a need for global techniques for analyzing the functions of genes. Transposons have long been recognized as powerful tools for inserting sequencing primer binding sites and for creating gene "knockouts" (insertional mutagenesis) in microorganisms. However, traditional

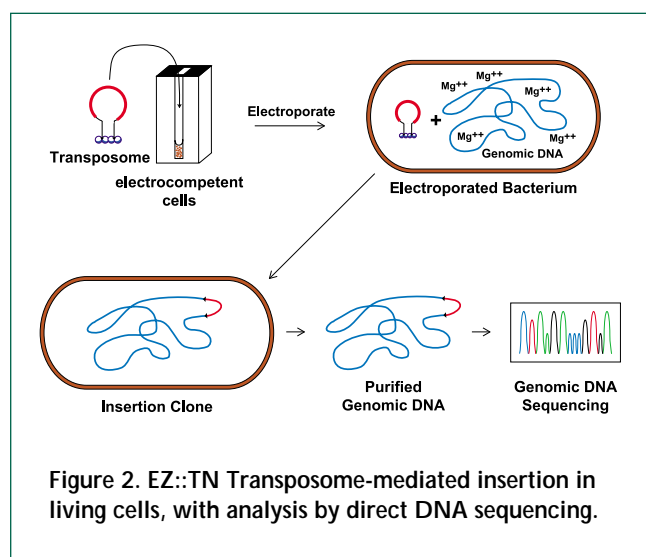
transposition systems are often difficult to work with and limited in the number of microbial species in which they can be used. EZ::TN Transposomes, based on the pioneering work of Reznikoff and Goryshin,<sup>2,3</sup> offer a new, faster, simpler and more efficient method to randomly introduce transposons into the genomes of many different microorganisms.

An EZ::TN Transposome is a stable synaptic complex formed between the hyperactive Tn5 transposase and a Tn5-derived transposon. Although not required, the transposons used typically contain a selectable marker (e.g. antibiotic resistance gene) which allows rapid selection of transposed cells. Although EZ::TN Transposomes are normally formed transiently during transposition, a stable Transposome can be formed and isolated in the absence of Mg<sup>2+</sup> (Figure 1).



continued

EZ::TN Transposomes are stable enough to be electroporated into living cells<sup>3,4</sup> (Figure 2). Once inside the cell, the Transposome is activated, presumably by the  $Mg^{2+}$  within the host's cellular environment. Once activated, the EZ::TN Transposome efficiently and randomly inserts its transposon into the genomic DNA of the host cell. By screening antibiotic-selected transposition clones for a desired phenotype, gene knockouts in many open reading frames (ORFs) can be found. Thus, EZ::TN Transposomes eliminate the need for transductions or matings to mobilize transposons. In this report, we extend the use of EZ::TN Transposomes to randomly create insertion mutants in *Salmonella typhimurium*, *Proteus vulgaris* and *Pseudomonas sp.* Importantly, we also demonstrate that EZ::TN Transposomes facilitate direct DNA sequencing of mutated bacterial genomic DNA.



**Figure 2. EZ::TN Transposome-mediated insertion in living cells, with analysis by direct DNA sequencing.**

## Methods

### Transposon mutagenesis

EZ::TN <KAN-1>Tnp Transposomes were formed using purified, hyperactive EZ::TN Transposase and an EZ::TN Transposon containing a kanamycin-selectable marker. The standard reaction for EZ::TN <KAN-1>Tnp Transposome formation is a mixture of 5  $\mu$ l of 100  $\mu$ g/ml <KAN-1> Transposon in 10 mM Tris-HCl, pH 7.5; 1 mM EDTA, 10  $\mu$ l of 1 U/ $\mu$ l EZ::TN Transposase and 5  $\mu$ l of 100% glycerol. This procedure can be scaled up or scaled down as needed. EZ::TN <KAN-1>Tnp Transposomes were formed by incubating the mix at 37°C for 10 minutes. One-microliter aliquots were used for electroporation. The remaining EZ::TN <KAN-1>Tnp Transposome can be stored for up to 1 year at -20°C without loss of activity.

### Cell electroporation

*Proteus vulgaris* was obtained from ATCC (number 13,315) and *Pseudomonas sp.* (MMSS-8 strain) was from the University of Wisconsin Bacteriology Department

Stock Culture Collection. Electrocompetent cells were prepared in the same manner for all species. Cells were grown to mid-log phase at 37°C in LB broth with shaking, then chilled, harvested by centrifugation and washed with deionized water three times before suspending them in ice-cold 10% glycerol in deionized water. Cells were stored frozen at -70°C in 100- $\mu$ l aliquots until used.

Prior to use, cells were thawed on ice. Fifty microliters were transferred to a 2.0-mm gap electroporation cuvette. One microliter of EZ::TN <KAN-1>Tnp Transposome was added and the cells were electroporated at 2500 V and 5 microseconds time constant using an Eppendorf Multiporator. Transposition clones were selected by plating on LB plates containing 50  $\mu$ g per ml of kanamycin for *S. typhimurium* and *P. vulgaris* or 300  $\mu$ g per ml of kanamycin for *Pseudomonas sp.*

### Bacterial DNA isolation

Individual Kan<sup>r</sup> transposition clones were grown overnight at 37°C in LB Broth containing 50  $\mu$ g/ml kanamycin for *S. typhimurium* and *P. vulgaris* and 300  $\mu$ g/ml kanamycin for *Pseudomonas sp.* Genomic DNA was purified using the MasterPure™ Complete DNA Purification Kit (EPICENTRE).

### Direct genomic DNA sequencing and sequence analysis

Transposon insertion sites were sequenced bidirectionally using sequencing primers specific for the ends of the inserted transposon. Two to three micrograms of bacterial genomic DNA and 5-12 pmoles of primer were used in "2X" Big Dye Terminator sequencing reactions according to the manufacturer's protocols (PE Biosystems, Foster City CA). Bacterial genomic DNA does not require restriction endonuclease digestion or shearing to serve as a DNA sequencing template. Samples were cycled (DNA Engine, MJ Research, Waltham, MA) for 4 min at 95°C, then 60 cycles of 30 sec at 95°C and 4 min at 60°C followed by 4°C indefinitely. Sequencing reactions were purified by gel filtration with a Centri-Sep spin column (Princeton Separations, Princeton, NJ), concentrated by ethanol precipitation, washed with 70% ethanol, and resuspended in 20  $\mu$ l of Template Suppression Reagent (PE Biosystems, Foster City, CA). After denaturing at 95°C for 5 min, the samples were injected into an ABI 310 Genetic Analyzer (PE Biosystems, Foster City, CA) and analyzed with ABI version 3.3 sequence analysis software. Transposon insertion sites can also be sequenced using radioactive and LI-COR sequencing methods (Ronald Meis, EPICENTRE, unpublished data). The genomic transposition sites were located using BLAST programs maintained at the NCBI web site of the National Library of Medicine.

The primer EB-L was used for sequencing 16S rDNA.<sup>5</sup> Cycle sequencing and sample preparation and analysis were the same as above.

## Results and Discussion

As demonstrated previously,<sup>3,4</sup> EZ::TN Transposomes can be electroporated into *E. coli* and yeast and lead to transposon integration into chromosomal DNA. DeAngelis<sup>6</sup> recently described a method for direct transposon-primed sequencing of bacterial genomic DNA which entails use of restriction endonuclease digestion and size selection of genomic fragments. In this report we extend the list of species successfully transposed by EZ::TN Transposome technology. We also describe simplified and improved techniques for inserting, locating and sequencing the disruption sites of introduced transposons.

### EZ::TN Transposome-mediated transposon insertion

Electroporation of EZ::TN Transposomes into cells eliminates the need for matings and suicide vectors for transposon insertions into the genome of microorganisms. To demonstrate the applicability of the EZ::TN Transposome technology to a wide variety of microorganisms, we show here that *Pseudomonas sp.*, *Salmonella thyphimurium* and *Proteus vulgaris* can be successfully and stably transposed as determined by their resistance to kanamycin (Table 1). Electroporation of EZ::TN Transposomes was found to vary in efficiency from one bacterial host to another. The variation of efficiency is most likely due to use of sub-optimal electroporation conditions.

<i>E. coli</i>	<i>Salmonella typhimurium</i>	<i>Proteus vulgaris</i>	<i>Pseudomonas sp.</i>
8.4 x 10 <sup>6</sup>	5.6 x 10 <sup>5</sup>	1.0 x 10 <sup>5</sup>	5 x 10 <sup>3</sup>

**Table 1.** Average number of transposition clones generated by electroporation of EZ::TN <KAN-1>Tnp Transposome. All values are in number of Karf colonies per µg DNA.

One interesting finding was a readily-recognized phenotype change to one *P. vulgaris* transposition clone. *P. vulgaris* is normally a motile organism that demonstrates a "swarming" growth phenotype on agar plates. One of the *P. vulgaris* transposition clones was unable to swarm indicating that a gene knockout had probably been created in this clone.

### Direct genomic DNA sequencing of transposon insertion sites

Genomic DNA from four *P. vulgaris* transposition clones, including the non-swarming clone, was purified and directly sequenced as described in *Methods*. Two to 2.5 µg of *P. vulgaris* DNA were sufficient to obtain sequence reads of approximately 400 bases (Fig. 3, p. 4). Using the primers from each end of the transposon, nearly a kilobase of sequence could be read from a single transposition clone.

Clone	Genetic Symbol of Interrupted Loci
Proteus (α)	23S Ribosomal RNA
Proteus (β)	tRNA phe
Proteus (γ) *	fimbrial operon <i>atf</i>
Proteus (δ)	<i>yhdG</i> homolog

\* non-swarming phenotype observed

**Table 2.** Identification of transposon insertion sites in *Proteus vulgaris* transposed by EZ::TN <KAN-1>Tnp Transposome.

BLAST homology analysis of genomic sequence data generated from the four *P. vulgaris* transposition clones identified the specific transposon insertion site of each (Table 2). Two were identified by direct matching to *P. vulgaris* sequences and one by BLAST homology to an *E. coli* tRNA gene. The tRNA<sup>phe</sup> gene is highly conserved between *E. coli* and *P. vulgaris*, but sequence homology diverges rapidly beyond the coding region of the tRNA. Another transposon landing site was a homolog of the *nifR3* gene of *Rhodobacter*, which is induced in response to nitrogen limitation. The third transposition clone had an interruption of a large subunit ribosomal RNA (rRNA) gene. There are probably seven or more copies of the rRNA gene in *P. vulgaris*.

The transposon insertion in the non-swarming mutant clone of *P. vulgaris* was discovered to be within its fimbrial gene (pilus protein) homolog, *atf*, one of at least 45 genes involved in the *Proteus* swarming phenotype.<sup>7</sup> Thus, EZ::TN Transposome disruption of the pilus protein gene would explain the loss of the swarming phenotype. Our results demonstrate rapid production of a gene knockout and correlation of cell phenotype with the genomic locus by direct genomic DNA sequencing.

Direct genomic sequencing of several randomly-selected *Pseudomonas sp.* transposition clones led to the identification of the chromosomal locations of several of the clones. Interestingly there was no complete homology with the complete *Pseudomonas aeruginosa* sequence for any of six transposition clones sequenced. One explanation was that the bacterium was not *P. aeruginosa* as it had been designated. The sequences of the 16S ribosomal RNA genes of many bacteria are known and may be used to classify them taxonomically. We used direct genomic DNA sequencing from a 16S rDNA primer to tentatively identify the species named *P. aeruginosa* in the strain collection. The best match of 16S rDNA was to a polycyclic aromatic hydrocarbon-degrading *Pseudomonas* species originally found in creosote-contaminated soils.<sup>8</sup>

continued

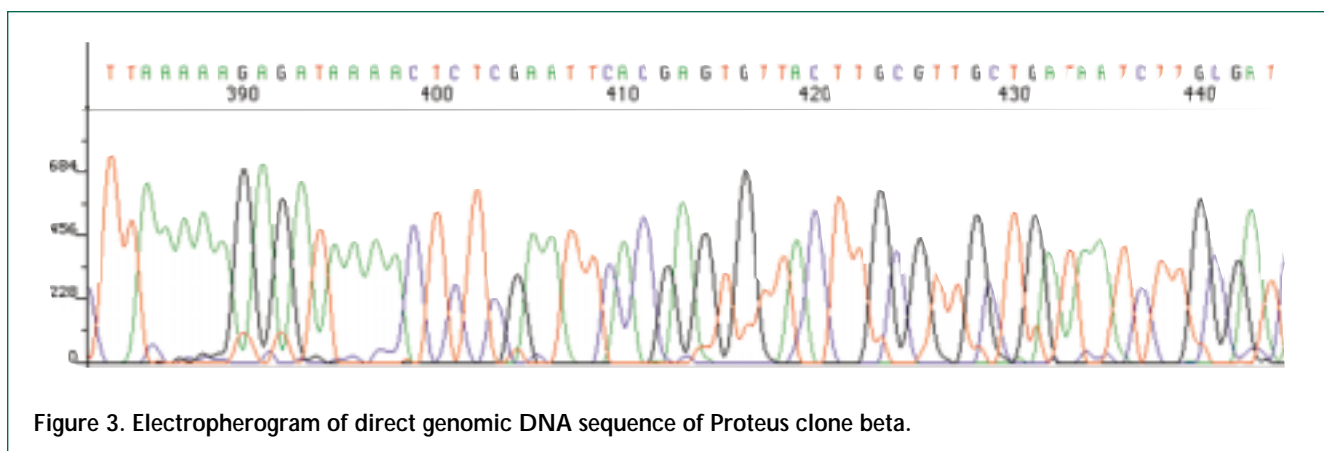


Figure 3. Electropherogram of direct genomic DNA sequence of *Proteus* clone beta.

Its 16S rDNA sequences are 87-95% homologous to *P. aeruginosa* sequences, and except for antibiotic sensitivities, other properties of the bacterium are not known.

Identifying the MMSS-8 bacterium as a non-*P. aeruginosa* species made the localization of transposon insertion sites more ambiguous. The strongest homologies were to the *P. aeruginosa* MexE gene involved in efflux systems responsible for low level multidrug resistance<sup>9</sup> and in another clone for the P16 subunit of the MvaT transcriptional activator.<sup>10</sup> The Mva operon is involved in isoprenoid synthesis in pseudomonads, other gram negative bacteria and mycobacteria. The synthetic pathway for isoprenoids is absent in vertebrates and man and may present a useful target for antibacterial drugs.

### Conclusions

EZ::TN Transposome technology is a unique, simple means of introducing any DNA segment into bacterial chromosomes. This new technology allows the *in vivo* insertion of transposons into several bacterial species. In fact, with improvements in the efficiencies of bacterial electroporation, there is reason to believe that *in vivo* Transposome-mediated mutagenesis with EZ::TN Transposomes could be applied to any bacterium for which a selectable marker exists. Not only is the EZ::TN Transposome-mediated transposon insertion process easy and fast, but the inserted transposons facilitate rapid analysis of the transposon insertion sites by such means as direct genomic DNA sequencing.

The power of EZ::TN Transposomes is further expanded by the availability of an EZ::TN pMOD™<MCS> Transposon Construction Vector\* that allows the user to construct an EZ::TN Transposon containing any DNA sequence of interest (e.g. selectable markers, genetic control elements).

### Acknowledgements

Thanks go to Mr. John Lindquist of the University of Wisconsin-Madison Department of Bacteriology for the *Pseudomonas* sp. culture. We are indebted to Drs. Igor Goryshin and William Reznikoff at the University of

Wisconsin-Madison Department of Biochemistry for introducing us to the multitudinous possibilities lying ahead for transposome-mediated gene hopping.

**Note:** The EZ::TN <KAN-1>Tnp Transposome used in this study has been replaced by the smaller, but functionally equivalent EZ::TN <KAN-2>Tnp Transposome. See the center insert for ordering information.

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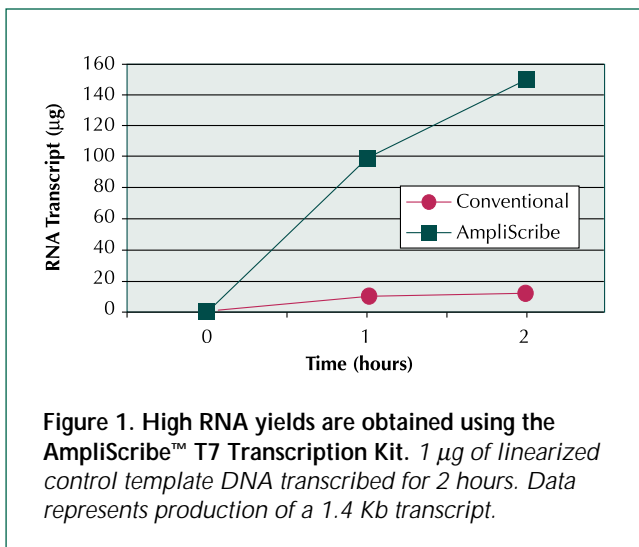
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# Ultra-Sensitive Purification of Microbial Nucleic Acids Using the MasterPure™ Complete DNA and RNA Purification Kit

John Watson, EPICENTRE Technologies

Nucleic acid amplification has become the method of choice for identification and typing of microorganisms. In many cases, the limiting factor for sensitive detection is the method used for extraction of the nucleic acid. The MasterPure Complete DNA and RNA Purification Kit is specifically designed to provide research laboratories with a rapid, sensitive, non-hazardous, and extremely simple method for isolating microbial DNA or RNA. The method uses a modified salt precipitation protocol that incorporates a co-precipitant\* that substantially improves sensitivity and permits the extraction of RNA and/or DNA from virtually any microbial sample.

The gold standard for nucleic acid extraction involves the removal of proteins from nucleic acids using organic solvents such as phenol and/or chloroform.<sup>1</sup> The hazardous nature of these methods has led to the development of column-based methods that eliminate the organic solvents. Unfortunately, column technologies introduce other problems including: contamination due to repeated contact with the columns; complicated manipulation of buffers while loading and washing the columns; frequent labeling and handling of microcentrifuge tubes; sensitivity problems due to non specific binding; and finally, "drop-out" issues where no nucleic acid is detected. What is needed is a method that combines the consistency of organic methods, with the safety of column methods.

In this article, we compare the MasterPure Complete DNA and RNA Purification Kit with a major manufacturer's spin column method for DNA extraction. We found that the spin column method resulted in reduced yields at low cell numbers relative to the MasterPure Complete Kit. At very low cells numbers, the column method produced no detectable DNA. These data suggest columns have non-elutable binding sites that reduce the sensitivity of the method.

## Methods

### *MasterPure Complete Protocol*

The MasterPure Complete technology is based on the salt precipitation method described by Miller *et al.*;<sup>2</sup> specific protocols are available on our web site at [www.epicentre.com/lit/mpclit.htm](http://www.epicentre.com/lit/mpclit.htm). Twenty  $\mu$ l samples from serial 10-fold dilutions of a liquid *E. coli* strain MC1061-lambda (containing  $2 \times 10^7$  to 200 organisms per 20  $\mu$ l sample) were processed as follows. Bacteria were added to 300  $\mu$ l of Tissue and Cell Lysis Buffer (RNase A was added for DNA-only samples) and treated with

proteinase K. After chilling on ice, 160  $\mu$ l of Protein Precipitation Reagent were added and the debris pelleted. The supernatant was poured into a fresh microcentrifuge tube and the nucleic acid precipitated with isopropanol. After washing with 70% ethanol, the pellet was resuspended in 10  $\mu$ l of sterile water. DNA concentrations were assayed using a Hoefer DyNA Quant™ fluorimeter and Hoechst 33258 fluorescent dye. Purity of nucleic acids was assayed by spectrophotometry.

### *Spin Column Method*

The spin column method was performed according to the manufacturer's instructions. The same dilution series described above was processed as follows. Twenty  $\mu$ l of diluted cells were pelleted and resuspended in the manufacturer's cell lysis solution and treated with proteinase K. After loading and washing the spin column, the DNA was eluted in 50  $\mu$ l of sterile water as suggested by the manufacturer for samples containing small amounts of DNA.

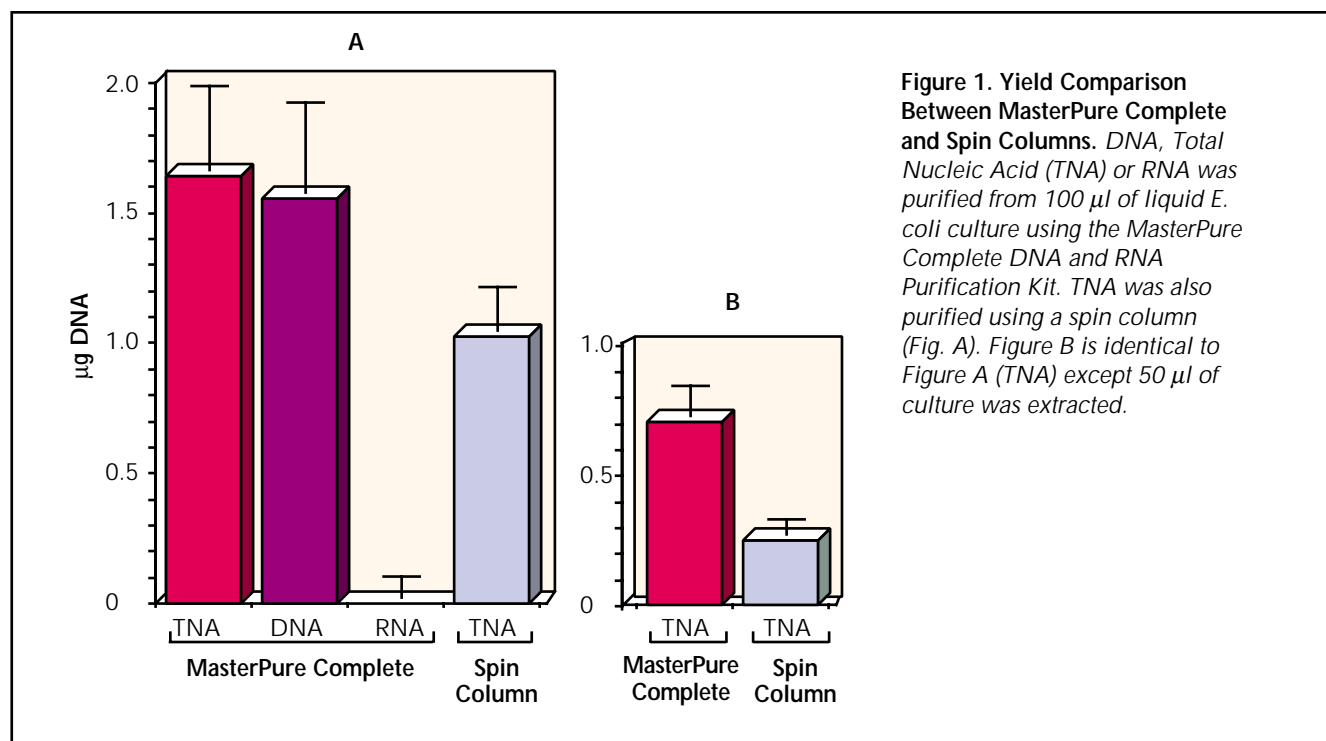
### *PCR Amplification*

A 188 bp region of an integrated lambda genome was amplified using the following conditions; PCR reactions contained 0.5  $\mu$ M of each primer (TACACAACCGCC-CAACTGC, forward primer; CGGGAACGGATAACCTCAC, reverse primer), 25  $\mu$ l of MasterAmp 2X PCR Buffer E (web address [epicentre.com/lit/maoptlit.htm](http://www.epicentre.com/lit/maoptlit.htm)), 0.25  $\mu$ l MasterAmp™ Taq Thermostable DNA Polymerase (<http://www.epicentre.com/lit/taqlt.htm>); and water to 50  $\mu$ l total volume. Cycling conditions included a denaturation step at 94°C for 1 min., followed by 30 cycles of: 94°C for 1 min.; 60°C for 1 min., 72°C for 1 min. Amplification products were resolved on 2% agarose gels and stained with ethidium bromide.

## Results and Discussion

### *Yield Comparisons*

The advent of spin columns provided an alternative to organic solvents for the extraction of nucleic acids. While spin columns have substantial problems, such as limited binding capacity and cumbersome protocols, these disadvantages have not prevented columns from becoming a popular method for nucleic acid purification because they eliminate hazardous solvents. Other potential problems with spin columns are non-elutable binding and dead volumes that may limit sensitivity. Most nucleic acid purification protocols were developed under situations where the amount of starting material was not limiting.



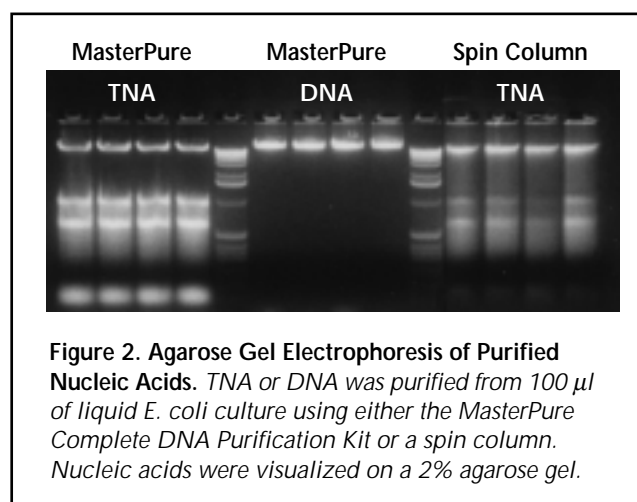
**Figure 1. Yield Comparison Between MasterPure Complete and Spin Columns.** DNA, Total Nucleic Acid (TNA) or RNA was purified from 100 µl of liquid *E. coli* culture using the MasterPure Complete DNA and RNA Purification Kit. TNA was also purified using a spin column (Fig. A). Figure B is identical to Figure A (TNA) except 50 µl of culture was extracted.

Many research laboratories and essentially all clinical microbiology laboratories, however, need extraction protocols that give the highest sensitivity possible.

We have recently developed a technology for nucleic acid isolation that combines the simplicity and sensitivity of non-column based methods with the safety of non-organic methods. The MasterPure Complete DNA and RNA Purification Kit uses a strong ionic detergent (and proteinase K, if necessary) to lyse cells and high salt to precipitate contaminating proteins. Nucleic acids are then concentrated by isopropanol precipitation, which allows resuspension in any volume necessary to assure sensitive detection.

This study was designed to determine the yield of DNA from small samples and at the low concentrations of microorganisms. Figure 1 compares the DNA yields obtained with the MasterPure Complete DNA and RNA Purification Kit versus the spin column method. The MasterPure Complete protocols for the isolation of total nucleic acid (TNA) and DNA are identical except the DNA samples are treated with RNase A to eliminate intact RNA. Figure 1A shows that the yield of DNA (as assayed by fluorimetry, which is insensitive to RNA) from  $3.5 \times 10^8$  cells is similar using either the DNA or TNA protocol. The spin column method yields substantially less DNA relative to either of the MasterPure Complete protocols. Specifically, the yield of DNA using the TNA protocol was 63% lower (0.53 µg less) using the spin column method than with the MasterPure Complete protocol. To help clarify the reason for lower yields with the spin column method the experiment was repeated

with half the number of cells. As shown in Figure 1B the column method yielded 0.46 µg less DNA. This suggests that the difference is due to non-elutable binding of approximately 0.5 µg of DNA to the column. Nucleic acids purified by the two methods were analyzed by agarose gel electrophoresis and stained with ethidium bromide (Figure 2). The TNA and spin column methods purify both RNA and DNA. Treatment with RNase A in the

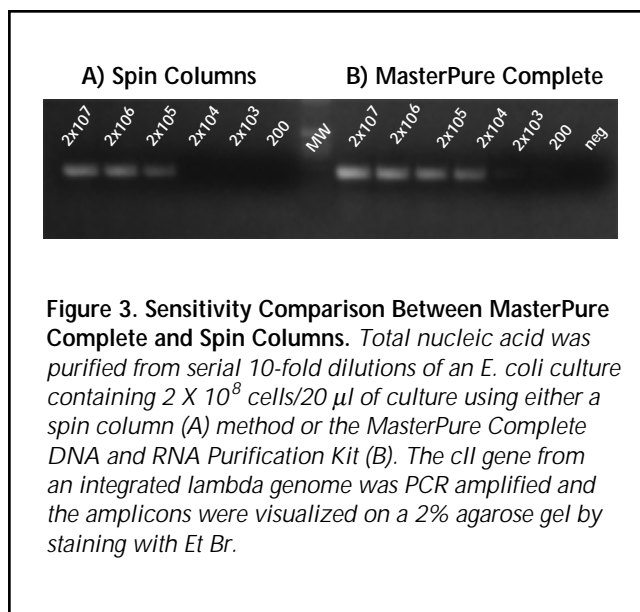


MasterPure DNA protocol eliminates the intact RNA.

The reduced DNA yield using the spin column method suggested that the MasterPure method may be more sensitive for extraction from small numbers of organisms. To investigate whether this is the case, DNA was extracted

*continued*

from 10-fold serial dilutions of an overnight culture of *E. coli* cells. The purified nucleic acid was used to amplify a region of the lambda *cII* gene. Consistent with the yield data, the spin column method was substantially less



sensitive in extracting DNA from small numbers of cells (Figure 3).

### Conclusion

As molecular technology evolves away from first generation extraction protocols, new methods must be developed that permit rapid learning and optimization. Older technologies for extracting DNA, like phenol-chloroform and spin column methods, were not designed to be rapid or particularly simple. In contrast, the MasterPure Complete DNA and RNA Purification Kits were specifically conceived and developed for easy use. The method is useful for the full range of samples extracted by research or clinical laboratories, including samples containing extremely low numbers of organisms.

### References

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# The New MasterAmp™ High Fidelity RT-PCR Kit for Accurate and Flexible RT-PCR

Judith T. Schanke, EPICENTRE Technologies

## Introduction

Reliable and accurate amplification of RNA templates is crucial for research based on the cloning and expression of new messages. Reverse transcription and subsequent PCR amplification (RT-PCR) are both error-prone enzymatic reactions.<sup>1,2</sup> The use of a high-fidelity enzyme blend containing a thermostable DNA polymerase with 3' → 5' exonuclease (proofreading) activity is critical when the accuracy of the amplification product is important.

Here we introduce the MasterAmp™ High Fidelity RT-PCR Kit, the most convenient high-fidelity RT-PCR system available. The RT or first-strand cDNA synthesis is catalyzed by Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT) Plus, the most reliable enzyme for full-length cDNA synthesis.<sup>3</sup> Subsequent PCR uses MasterAmp TAQurate™ DNA Polymerase Mix, a unique blend of thermostable DNA polymerases that dramatically reduces the error rate of PCR amplification. The kit also includes a convenient MasterAmp 2X RT-PCR PreMix containing reaction buffer, dNTPs and MgCl<sub>2</sub> that has been optimized for both reverse transcription and PCR amplification.

The MasterAmp High Fidelity RT-PCR Kit provides flexibility when performing RT-PCR reactions as either a one-step or two-step procedure (Figure 1). The one-step procedure is designed for performing first-strand synthesis and PCR in one tube using specific primers supplied by the user. Since reverse transcription and PCR are carried

out successively without opening the tube lid, this protocol minimizes sample handling and the possible introduction of Rnases.

In the two-step procedure first-strand synthesis is performed using either random nonamers or oligo d(T)<sub>18</sub> primers supplied in the kit. Subsequent PCR is achieved in one of two ways. The modified two-step procedure, shown in Figure 1B, allows the addition of specific primers and TAQurate DNA Polymerase Mix directly into the reaction mix after the RT step, eliminating the need to set up a separate PCR reaction. The standard two-step RT-PCR can also be performed. In this protocol, a small amount (5-20%) of the RT reaction is removed for subsequent PCR. This variation can be useful when several different primer pairs are used to analyze the same cDNA template.

In this article we demonstrate amplification from a variety of different RNA templates, including multiple targets, using the MasterAmp High Fidelity RT-PCR Kit. We also show RNA amplification using non-specific RT primers and amplification of long RNA templates.

## Materials and Methods

The standard one-step RT-PCR reactions contained 100-500 ng total cellular or viral RNA, 12.5 pmoles of each specific primer, 1X RT-PCR PreMix, 40 Units of MMLV-RT Plus, and 1 U of TAQurate DNA Polymerase Mix in a total volume of 50 µl. Reverse transcription was performed at 37-50°C for 30-60 minutes, depending on the secondary structure and length of the RNA template. The reverse transcription was immediately followed by a denaturation step at 95°C for 1 minute, then 30 cycles of 92°C for 30 seconds, 56°C for 30 seconds, and 72°C for 30 seconds to 6 minutes, depending on the length of the expected product. The annealing step for the amplification of tobacco mosaic virus (TMV) RNA was increased to 62°C.

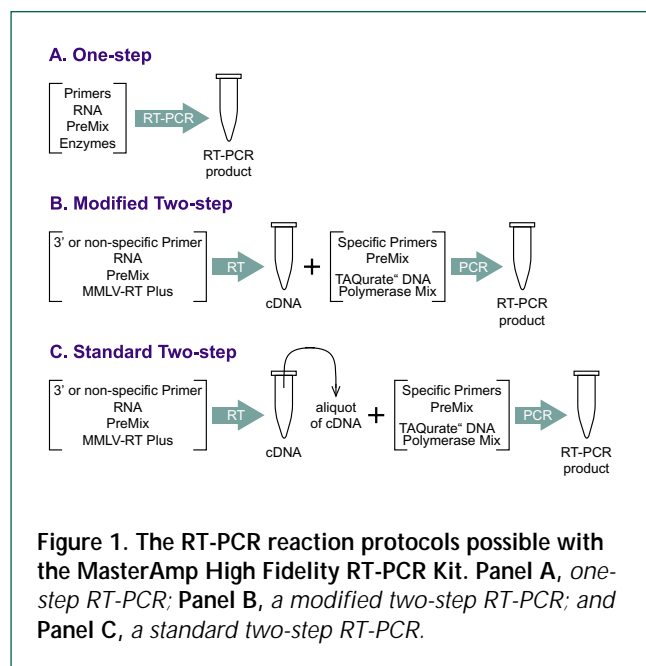
The modified two-step amplifications included 0.5 µg random hexamer oligonucleotides or 25 pmoles of oligo (dT)<sub>18</sub> during the reverse transcription reaction and were incubated for 30 minutes at 37°C. After first strand synthesis, 12.5 pmoles of each specific primer were added and the reactions were cycled as described previously.

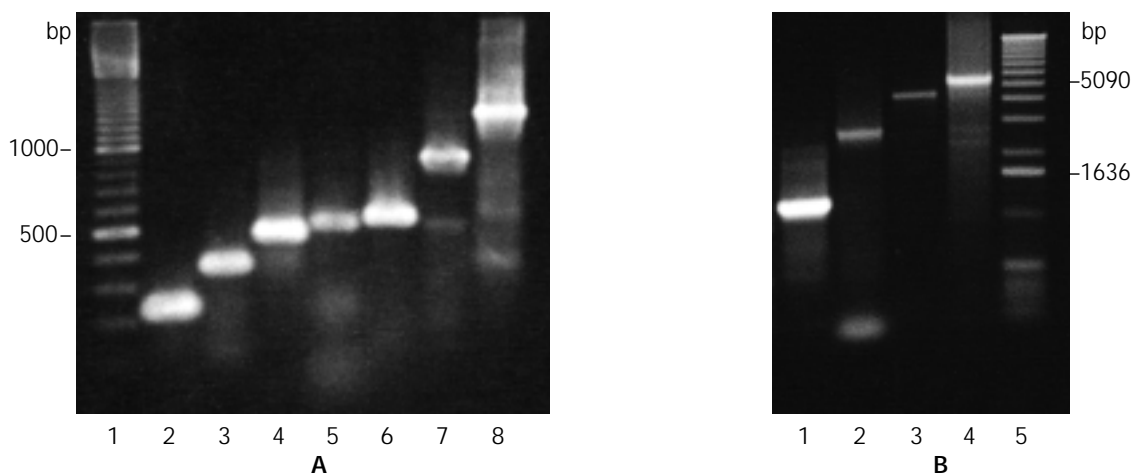
## Results and Discussion

### *Efficient amplification of a variety of RNA templates*

RNA templates used for RT-PCR vary in complexity, structure, and length. Figure 2A demonstrates the amplification of messages from sources including total cellular human, bacterial, and viral RNA preparations.

*continued*





**Figure 2. Efficient amplification of a variety of RNA templates using the MasterAmp High Fidelity RT-PCR Kit. Panel A:** Lane 1, 100 bp ladder; Lane 2, 250 bp human lung  $\beta$ -actin; Lane 3, 350 bp human lung GAPDH; Lane 4, 463 bp region of TMV; Lane 5, 479 bp *E. coli* 16s rRNA; Lane 6, 589 bp human placental CG $\alpha$ ; Lane 7, 850 bp human heart  $\beta$ -actin; and Lane 8, 1248 bp region of TMV. **Panel B:** Long amplifications of TMV RNA. Lane 1, 1,248 bp; Lane 2, 2,795 bp; Lane 3, 4,517 bp; Lane 4, 5,503 bp; and Lane 5, kb ladder.

Figure 2B demonstrates the ability of the MasterAmp High Fidelity RT-PCR Kit to amplify full-length RT-PCR product from specific regions of TMV RNA. RT-PCR amplifications were all performed using the convenient one-step protocol (Figure 1A) with template-specific primers for 30 cycles. No reaction optimization was required to obtain substantial yields from each of these targets.

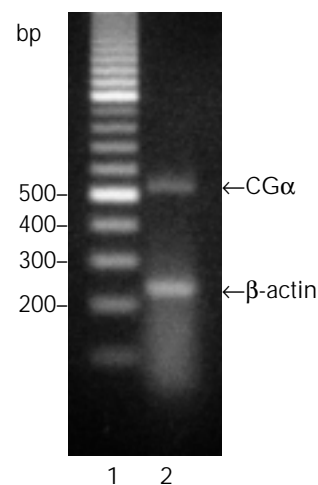
The simultaneous amplification of more than one RNA target or multiplex amplifications can also be performed in a single tube using the standard one-step protocol. Figure 3 shows the co-amplification of chorionic gonadotropin (CG) $\alpha$  and  $\beta$ -actin from human placental RNA.

#### *Specific amplification using the modified two-step protocol*

A modified two-step procedure is demonstrated in Figure 4. Reverse transcription was performed on cellular RNA from human lung with either random hexamers or oligo d(T)<sub>18</sub> primers.  $\beta$ -Actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sequences were then amplified by the addition of TAQurate DNA polymerase mix and gene specific primers. As a comparison, the specific rather than random primers, were used for first-strand synthesis. All three reactions produced a high yield of specific product with this modified two-step protocol.

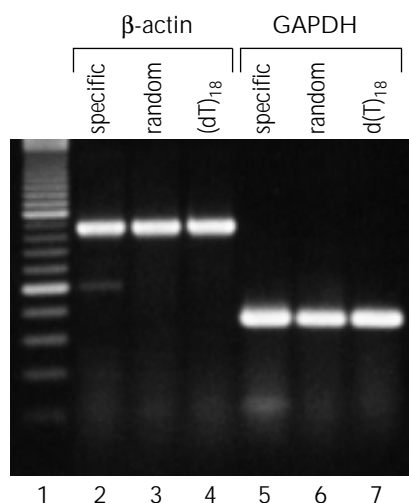
#### *Full-length amplification products from long RNA templates*

Standard RT-PCR kits can not be used to adequately produce full-length cDNAs from targets longer than 3kb. Figure 5 (lane 2) demonstrates that a 5.5kb segment of TMV RNA, containing complex secondary structure, was only amplified with high specificity using the MasterAmp High Fidelity



**Figure 3. Multiplex RT-PCR using the MasterAmp High Fidelity RT-PCR Kit.** Lane 1, 100 bp ladder; Lane 2, co-amplification of 589 bp CG $\alpha$  and 250 bp  $\beta$ -actin from human placental RNA.

RT-PCR Kit. Although the one-step reaction procedure was used, a two-step amplification also produced specific, full-length product (not shown). The same thermal cycling parameters were used in a standard two-step amplification with MMLV-RT and a standard Taq DNA polymerase. Not using the MasterAmp blend of thermostable DNA polymerases resulted in a small amount of full-length product, but also produced numerous, undesirable, smaller amplification products (Figure 5, Lane 3). A one-step,



**Figure 4.** The modified two-step protocol using the MasterAmp High Fidelity RT-PCR Kit to amplify RNA reverse transcribed with non-specific primers. Total cellular RNA purified from human lung tissue was reverse transcribed with either message-specific (Lanes 2 & 5), random hexamer (Lanes 3 & 6), or oligo (dT)<sub>18</sub> (Lanes 4 & 7) primers prior to PCR with message-specific primers for  $\beta$ -actin or GAPDH.

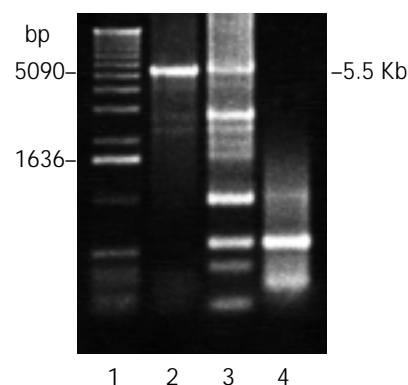
high-temperature reverse transcription with MasterAmp Tth DNA polymerase performed at 60°C for 60 minutes with identical PCR cycling conditions, resulted in no detectable full-length product (Figure 5, Lane 4). These results show that the MasterAmp High Fidelity RT-PCR Kit is a more reliable and specific approach for the accurate amplification of long regions of RNA.

### Summary

The MasterAmp High Fidelity RT-PCR Kit accurately and conveniently reverse transcribes and amplifies cDNA from RNA templates. The amplification of sequences from a variety of RNA templates was demonstrated, including the ability to amplify long templates with better efficiency and specificity than standard methods. One-step or two-step amplifications with random, oligo dT, or specific primers can all be performed with the MasterAmp High Fidelity RT-PCR Kit. This protocol flexibility, along with the MasterAmp 2X RT-PCR PreMix and the use of the TAQurate DNA Polymerase Mix, makes the MasterAmp High Fidelity RT-PCR Kit the most versatile and convenient high-fidelity RT-PCR Kit for cloning and expression of cDNA.

### References

1. Bebenek, K. *et al.* (1993) *Reverse Transcriptase*, Cold Spring Harbor Laboratory Press, 85.
2. Cline, J. *et al.* (1996) *Nucl. Acids Res.* **24** (18), 3546.
3. Johnson, M. (1996) *Epicentre Forum* **3** (3), 8.



**Figure 5.** The MasterAmp High Fidelity RT-PCR Kit more efficiently amplifies full-length products from long RNA templates. RT-PCR products from TMV RNA with primers that amplify a 5.5 kb region of the viral RNA. Lane 1, kb ladder; Lane 2, amplification using the convenient one-step protocol; Lane 3, amplification with a standard two-step procedure using MMLV-RT and Taq DNA polymerase; Lane 4, amplification with Tth DNA polymerase.

### MasterAmp™ High Fidelity RT-PCR Kit

25 Reactions	RF91025
100 Reaction	RF910100

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### EPICENTRE FORUM

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Finally, EPICENTRE's MasterAmp PCR Enhancement Technology (with betaine)<sup>+</sup> can substantially improve the yield and specificity of amplification reactions, especially where the template has a high G+C content or secondary structure. Simply titrate as needed for particularly difficult amplifications.

<sup>+</sup>Patents issued and pending.

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## Comparison of EPICENTRE's MasterAmp™ RT-PCR Kits

Features:	<span style="color: red; font-weight: bold;">New</span> MasterAmp™ High Fidelity RT-PCR Kit	MasterAmp™ RT-PCR Kit* (for high sensitivity)
Highest Fidelity	✓	
Highest Sensitivity		✓
Enzymes	MMLV-RT Plus and MasterAmp™ TAQurate™ DNA Polymerase Mix	RetroAmp™ RT DNA Polymerase
Both One-Step and Two-Step Protocols	✓	✓
Includes MasterAmp™ Enhancer Technology	✓	✓
Includes Random/Oligo dT Primers	✓	✓
Longest Amplifications	✓	
High Temperature cDNA Synthesis		✓

*\*This is the original MasterAmp™ RT-PCR Kit*

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