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INTRODUCTION

Description

Intact and full-length cDNA is critical to the success of many RNA expression protocols such as library construction, RT-PCR, microarrays, GeneSystem320 (GS320) and differential display. KPL's cDNA Integrity Kit allows for the determination of the quality of cDNA prior to its use in other applications. Results can be obtained from any single or double-stranded cDNA synthesized from human, mouse or rat cells and tissue.

The cDNA Integrity Kit utilizes primer sets and target genes that allow evaluation of in-process or double-stranded cDNA for the presence of full-length and extended cDNA transcripts. The kit has been designed to amplify target cDNA using eight primer sets for four different genes under universal PCR conditions. The target genes were chosen based on their common occurrence in a wide range of cell types, the length of transcript and the high level of sequence conservation for the genes between species. Primer sets included in the kit amplify regions of the 3' and 5' ends of the housekeeping genes GAPDH and Beta-Actin, 3' and 5' ends of the low expressed ADP ribosylation factor I gene (ARF F1), and the 3' and 5' regions of the Clathrin gene, which has a transcript length of over 6 kb. Generation of product using the 3' primer sets indicate that the gene is expressed in your system. Amplimer production using the 5' primer sets indicate full length, intact cDNA. Primer sets for each gene are designed to have significant homology to human, mouse and rat species. Therefore, the use of one set of gene-specific primers across these species does not require individual optimization of each primer/template combination. Universal PCR conditions have been established to allow amplification of all 8 primer sets in a single thermal cycler run.

APPLICATIONS

Critical analysis of material used for cDNA synthesis as well as the resulting cDNA is necessary to eliminate pitfalls in downstream applications. Synthesis of cDNA that is fully representative of a mRNA population is essential for success with quantitative or relative analysis of gene expression and cDNA library generation. High quality RNA is necessary for synthesis of full-length cDNA. Variability associated with reverse transcriptase enzymes and protocols used in the reverse transcription reaction may lend itself to cDNA of varying quality. Both of these issues pose a problem when searching for unknown genes where a certain population of the cDNA may be under-represented. The cDNA Integrity Kit provides a method to assess the quality of the cDNA preparation prior to gene expression studies or library construction to ensure the presence of full-length transcripts.

This kit does not contain Taq polymerase, therefore, users are required to provide their own enzyme. The cDNA Integrity Kit has been successfully tested with a variety of thermostable DNA polymerases. We recommend Taq DNA Polymerase, native and recombinant (MBI Fermentas), Amplitaq (ABI), TaqBead (Promega) and Taq DNA Polymerase (Invitrogen Life Technologies). The cDNA Integrity Kit contains a proprietary PCR buffer (5X PCR Buffer, Catalog No. 601-0001) that has been designed for effective amplification of template using the 8 primer sets and the above mentioned polymerases under universal PCR conditions. Use of other DNA Polymerases with this kit will require additional optimization of components and cycling conditions based on the enzyme's characteristics. Please refer to individual supplier information for the recommended use of enzyme and buffer components.

Note: Purchase of this kit does not constitute a license for PCR. A licensed polymerase and licensed thermal cycler must be used in conjunction with this product. PCR is covered by patents owned by Hoffman-La Roche, Inc. and F. Hoffman-La Roche, Inc.

MATERIALS AND EQUIPMENT

<u>Kit Components</u>	<u>Product Code</u>	<u>Volume</u>
DEPC Treated Water	50-86-03	1.0 mL
5X PCR Buffer 1.5 mM MgCl ₂ , 200mM Tris-HCl (pH 8.3), 200 μM each (dATP, dGTP, dCTP, dTTP)	601-0001	720 μL
Control cDNA	601-0008	35 μL
GAPDH 3' Primer Set	601-0002	20 μL
GAPDH 5' Primer Set	601-0003	20 μL
Clathrin 3' Primer Set	601-0004	20 μL
Clathrin 5' Primer Set	601-0005	20 μL
Beta-Actin 3' Primer Set	601-0009	20 μL
Beta-Actin 5' Primer Set	601-0010	20 μL
ARF F1 3' Primer Set	601-0011	20 μL
ARF F1 5' Primer Set	601-0012	20 μL

All primer sets are supplied at a concentration of 200 ng/μL in TE Buffer.

Sufficient reagents are provided to perform 15 reactions plus 1 control reaction using all 8 primer sets with the control cDNA provided in the kit. Reagents must be stored at -20°C and kept on ice during use. Do not store kits in a frost-free freezer. The kit is stable for a minimum of 12 months from date of receipt when stored at -20°C.

REQUIRED SUPPLIES AND EQUIPMENT NOT INCLUDED

- RNA that has been reverse transcribed (first or second strand) or cDNA library
- Licensed DNA Thermostable Polymerase
 - Recommended: Taq DNA polymerase, native and recombinant (MBI Fermentas)
 - Amplitaq (Applied Biosystems)
 - Taq Bead (Promega)
 - Taq DNA Polymerase (Invitrogen Life Technologies)
- Licensed Thermal Cycler
- Gloves
- Micropipettors and sterile tips
- PCR tubes
- Ice bath
- Microcentrifuge
- Microcentrifuge tubes (autoclaved, DNase free)
- Electrophoresis equipment and apparatus
- UV transilluminator or fluorescent imager
- SYBR[®] Green I (BioWhittaker Catalog No. 50513) or Ethidium Bromide

PROTOCOL

PCR Reaction Set-Up

Note: The following protocol was optimized specifically for use with the eight primers sets and the 5X PCR Buffer included in the kit under set PCR cycling conditions. See the list above for recommended Taq Polymerases. Use of other thermostable polymerases and buffers may require additional optimization.

1. Allow all reagents to thaw completely on ice, vortex briefly and spin down in a microcentrifuge before pipetting. Keep all reagents on ice while in use. Pipette reagents slowly and carefully to avoid errors.
2. PCR reaction mix preparation:
 - A. Prepare a master mix on ice in a sterile microcentrifuge tube in the order as it appears below.

<u>COMPONENT</u>	<u>VOLUME PER REACTION</u>	<u>MASTER MIX*</u>
DEPC Water	variable	variable
5X PCR Buffer	5 μ L	45 μ L
Thermostable DNA Polymerase	variable (1 unit)	9 units
cDNA	variable: <ul style="list-style-type: none">• 1 – 10 ng for 1st or 2nd strand DNA• 0.5 μg cDNA library• Control cDNA (2 μL)	variable
Total Volume	24 μL	Total 216 μL

*Volume For 8 Reactions + 1 For Pipette Error

- B. Aliquot 24 μ L of the master mix to each PCR tube.
- C. Add 1 μ L of the appropriate primer set to each tube. Mix tubes with gentle mixing and centrifuge briefly. Proceed to thermal cycling.

PCR Cycling Conditions

	<u>TIME</u>	<u>TEMPERATURE</u>	<u>CYCLES</u>
Initial Denaturation	2 minutes	94°C	1
Denaturation	30 seconds	94°C	25
Anneal	30 seconds	55°C	
Extend	30 seconds	72°C	
Final Extension	7 minutes	72°C	1
Storage	Hold	4°C	

Analysis of PCR Products

Gel Electrophoresis and Staining

1. Run PCR products on a polyacrylamide or agarose gel.
 - a. For TBE polyacrylamide gels (4%, 4 - 12% or 4 – 20%), run 5 - 10 μ L of PCR product and the appropriate volume of loading dye.
 - b. For agarose gels (0.8 – 1%), run 15 - 20 μ L of PCR product and the appropriate volume of loading dye.
2. Stain the gel using a fluorescent DNA dye [e.g., Ethidium Bromide (0.5 μ g/mL) or SYBR Green I (1/10,000)] and visualize the PCR products using a UV transilluminator or fluorescent imager. Ethidium Bromide is not as sensitive as SYBR Green I, therefore, some products may appear faint with Ethidium Bromide staining.

Expected Results From High Quality cDNA

PRIMER SET	PCR PRODUCT SIZE (BP)
Clathrin 3'	700*
Clathrin 5'	570
ARF F1 3'	239*
ARF F1 5'	336
Beta-Actin 3'	720
Beta-Actin 5'	1000
GAPDH 3'	540
GAPDH 5'	887**

* For Rat species the 3' fragment of Clathrin and the 3' fragment of the ARF F1 gene may appear as weak bands on the gel. The primers sequences were derived primarily from human and mouse RNA information, resulting in a lower yield of these two products for rat samples. Some amplification of nonspecific products may also be observed.

** When using Human cDNA as template with the GAPDH 5' primer set, an additional PCR product (approximately 77 bp) may be observed. This should not affect the amplification or analysis of cDNA as long as the primary PCR product is present at 887 bp.

Figure 1. PCR from Human cDNA Synthesized from Dendritic Cell RNA.

Lane 1: MW marker 100 bp
Lane 2: Clathrin 3' product
Lane 3: Clathrin 5' product
Lane 4: ARF F1 3' product
Lane 5: ARF F1 5' product
Lane 6: Beta-Actin 3' product
Lane 7: Beta-Actin 5' product
Lane 8: GAPDH 3' product
Lane 9: GAPDH 5' product

5 ng Human cDNA was used as the template in PCR. 5 μ L of each PCR reaction were run on a 4% TBE Polyacrylamide Gel. The gel was stained with SYBR Green I and visualized using a fluorescent imager.

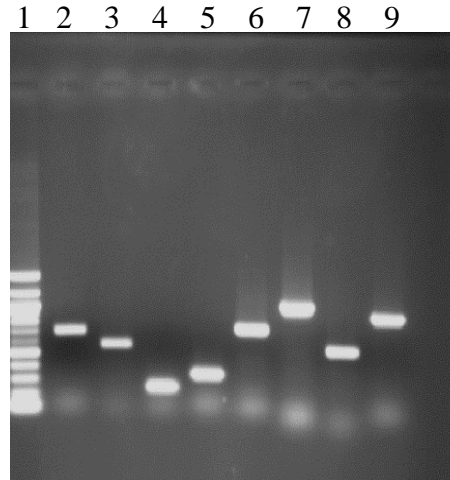
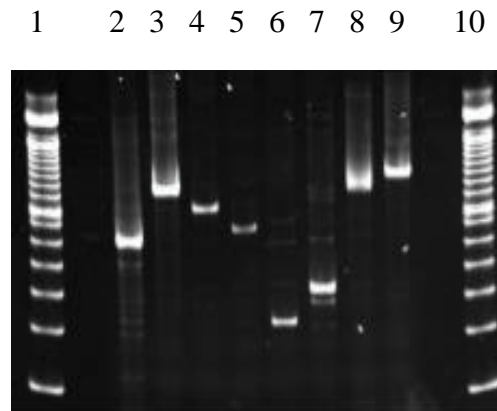


Photo Courtesy of John F. Foley, NIH, Bethesda, MD

Figure 2. PCR from Control cDNA (Rat)

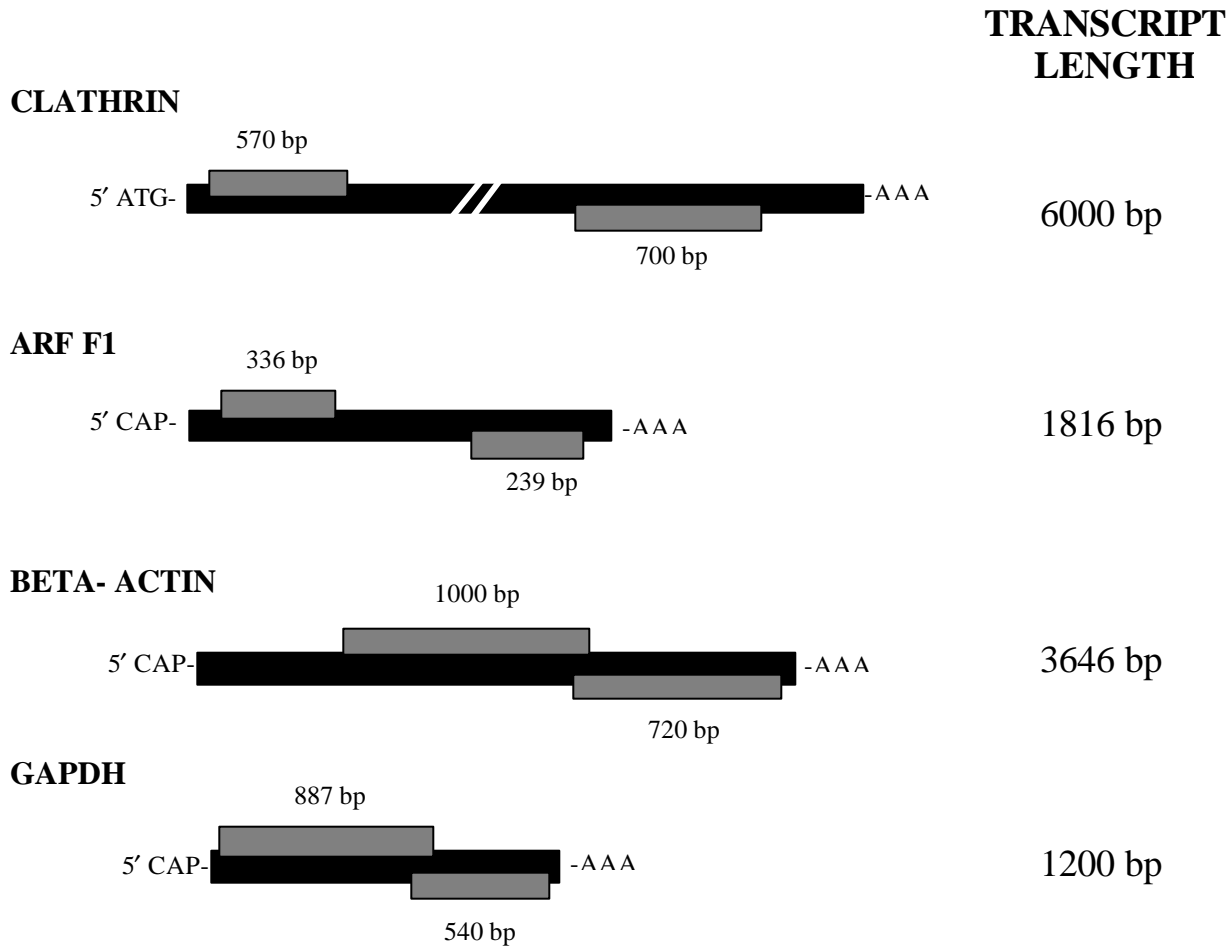
Lane 1: MW marker 100 bp
Lane 2: GAPDH 3' product
Lane 3: GAPDH 5' product
Lane 4: Clathrin 3' product
Lane 5: Clathrin 5' product
Lane 6: ARF F1 3' product
Lane 7: ARF F1 5' product
Lane 8: Beta Actin 3' product
Lane 9: Beta Actin 5' product
Lane 10: MW marker 100 bp

10 ng Rat Brain cDNA was used as the template in PCR. 5 μ L of each PCR reaction were run on a 4 – 12% TBE Polyacrylamide Gel. The gel was stained with SYBR Green I and visualized using a fluorescent imager.



MAP OF PRIMER SET PCR PRODUCTS

The relative location of each PCR product generated using the primer sets is illustrated below. The black areas indicate the relative length of the gene. The gray areas indicate the length of the amplified portion of the gene.



INTERPRETATION OF RESULTS AND TROUBLESHOOTING GUIDE

Problem	Possible Cause	Comments
No 3' product is observed.	<ul style="list-style-type: none"> • Degraded RNA/cDNA • Gene is not expressed in the test system. • PCR conditions not optimized. • cDNA concentration is too low. 	<ul style="list-style-type: none"> • cDNA is not full length or may not be intact. • Test for amplification of other genes included in kit. • Amplify control cDNA to determine if something is wrong with PCR conditions. • Titrate the amount of cDNA
No 5' product observed, but 3' product is present	<ul style="list-style-type: none"> • Degraded RNA/cDNA 	<ul style="list-style-type: none"> • cDNA is not full length or may not be intact
No product is observed in any of the reactions.	<ul style="list-style-type: none"> • A component was left out. • Insufficient amount of cDNA template or poor cDNA template quality. • Too much cDNA template added. • Too little thermostable polymerase used. • Thermostable polymerase incompatible with supplied buffer. • PCR Buffer is not thoroughly mixed. • Poor thermal cycler performance. 	<ul style="list-style-type: none"> • Use the control cDNA to validate the assay. • Insufficient amount of cDNA template may result in inefficient annealing of primers. Increase concentration of cDNA template or evaluate purity of template. • Too much cDNA template can be inhibitory to amplification. • 0.5 – 2.5 Units is sufficient for most applications. • Use a qualified supplier of thermostable polymerase (see page 3). • Vortex PCR Buffer immediately before use. • Check thermal cycler.
Multiple products and/or products that are smeared.	<ul style="list-style-type: none"> • Too much enzyme in the mixture. • cDNA template concentration too high. 	<ul style="list-style-type: none"> • Titrate the amount of enzyme (0.5 –2.5 units is usually sufficient). • Titrate the amount of cDNA template (too much template can be inhibitory).

RELATED PRODUCTS

<u>PRODUCT</u>	<u>SIZE</u>	<u>CATALOG NO.</u>
DNADetector™ HRP Southern Blotting Kit	20 blots	54-30-00
DNADetector™ Genomic Southern Blotting Kit	10 blots	54-30-03
RNADetector™ Northern Blotting Kit	20 blots	54-30-01
Detector™ Random Primer DNA Biotinylation Kit	30 reactions	60-01-00
Detector™ PCR DNA Biotinylation Kit	30 reactions	60-01-01
Detector™ RNA <i>in vitro</i> Transcription Biotinylation Kit	20 reactions	60-01-02
Biodyne® B Nylon Membrane	20 cm x 1 m	60-00-50
20X SSC	1 Liter	50-86-05
Hybridization Bags	50 bags	60-00-51
Herring Sperm DNA	40 mg	60-00-14
Spin-Pure Filters	5 filters	60-00-53

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