An Integrated Approach to Studying Apoptosis: From Gene Expression to Cellular Events

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We induced apoptosis in Jurkat cell cultures and measured DEVDase (caspase-3/7) activity using the Apo-ONE™ Homogeneous Caspase-3/7 Assay (Cat.#G7790, G7791). Total RNA was isolated from apoptotic and control cell cultures using the SV Total RNA Isolation System (Cat.# Z3100). This RNA provided the template for cDNA synthesis and gene-specific amplification using the ImProm-II™ Reverse Transcription System (Cat.# A3800). Together, these reagents allow a "systems approach" for studying apoptosis–from gene expression to cellular events.

Introduction

A number of specific changes in enzyme activity occur during apoptotic programmed cell death. The protease, caspase-3, increases in activity during apoptosis and participates in the degradation of several cellular proteins essential for DNA repair. The caspase-3 gene appears to be expressed throughout the life of the cell, and the cleavage of the inactive zymogen by upstream caspases is responsible for the increase in caspase-3 activity during apoptosis. We chose Jurkat cells as a model to demonstrate the induction of apoptosis and the associated molecular events. This article describes the application of Promega products for gene expression analysis, caspase assays, total RNA isolation, cDNA synthesis and DNA amplification to arrive at an integrated picture of apoptosis.

Cell Culture

We cultured Jurkat cells (~13 million cells per flask) in RPMI 1640 + 10% FBS in the presence of anti-Fas mAb to induce apoptosis. Control flasks (uninduced) were cultured in parallel using identical growth conditions but without the addition of the anti-Fas mAb.

Caspase-3/7 Assay

To confirm the induction of apoptosis, we used Promega's new Apo-ONE™ Homogeneous Caspase-3/7 Assay[a] (Cat.# G7790, G7791) to measure DEVDase (caspase-3/7) activity in the treated versus the control cell cultures following the protocol described in Technical Bulletin #TB295 (Figure 1). The Apo-ONE™ Homogeneous Caspase Assay is a homogeneous "add, mix, measure" assay for caspase-3/7 activity in cultured cells or in purified enzyme preparations (1).

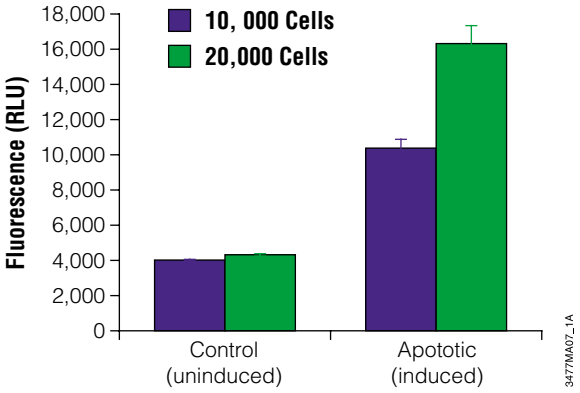

Figure 1. Caspase-3/7 activity of control and anti-Fas induced Jurkat cells. To induce apoptosis cells were suspended to 5×10^5/ml in RPMI 1640 + 10% FBS and treated with 100ng/ml of anti-Fas mAb for 4 hours at 37°C. Prior to isolating RNA, caspase-3/7 (DEVDase) activity in culture samples was measured using the Apo-ONE™ Homogeneous Caspase-3/7 Assay (Cat.# G7790, G7791).

RNA Isolation

We isolated total RNA from the cultured Jurkat cell suspensions using Promega's SV Total RNA Isolation System (Cat.# Z3100) according to the protocol in Technical Manual, #TM048. We divided the cultures into nine separate preparations of 1.4 million cells each, yielding approximately 180µg of total RNA for analysis of transcription levels from control and apoptotic cells.

Introducing Promega's new ImProm-II™ Reverse Transcription System

Promega's new ImProm-II™ Reverse Transcription System is a very efficient tool for gene expression analysis. The reaction products of ImProm-II™ cDNA synthesis readily adapt to a number of flexible options for analysis, and the system produces a high yield of full-length cDNA (2). Additionally, the optimized ImProm-II™ Reverse Transcription System reaction conditions enable robust Promega *Taq* DNA Polymerase[b] (Cat.# M1661) activity and are completely compatible with PCR methods. The ImProm-II™ Reverse Transcription System is optimized to allow choice of two-step, uncoupled RT and PCR or one-step, coupled RT-PCR (see methods).

RT-PCR from Jurkat cells using the ImProm-II™ Reverse Transcription System

We used control and apoptotic Jurkat total RNA as template in the first-strand cDNA synthesis following the protocol in Technical Manual #TM236. We denatured titrated amounts of RNA target and 0.5µg of oligo(dT)

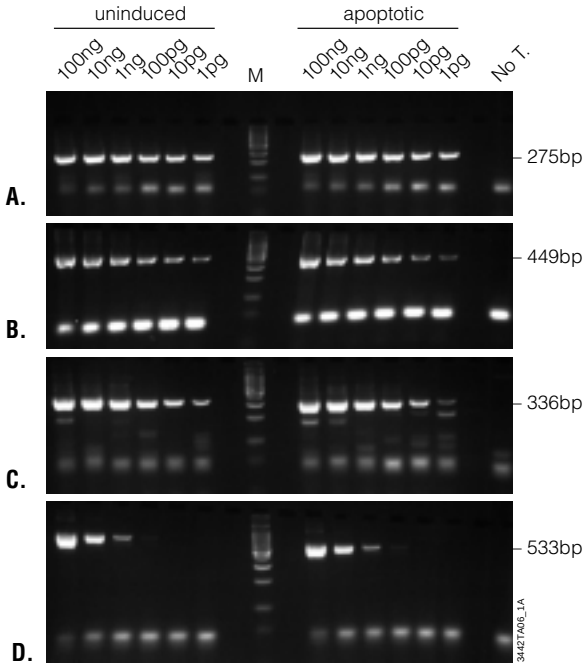

Figure 2. Efficient RT-PCR of mRNA for three housekeeping genes and caspase-3. Total RNA from apoptotic and uninduced Jurkat cells (Figure 1) isolated using the SV Total RNA Isolation System served as template in the first-strand cDNA synthesis using the Improm-II™ Reverse Transcription System. After we combined and denatured titrated amounts of RNA (1pg–100ng) and 0.5µg oligo(dT) primer, we assembled the reactions as described in Technical Manual #TM236. We performed uncoupled PCR on the resulting cDNA libraries by gene-specific amplification of target genes using the primers described in Table 1 (page 8). We visualized the products on a 4% agarose TBE gel by ethidium bromide staining. Markers are 100bp DNA Ladder. (Panel A, β-actin; Panel B, γ-actin; Panel C, ADP Ribosylation Factor-1; Panel D, Caspase-3).

primer. After denaturation, we assembled cDNA synthesis reactions.

To demonstrate the similarities between control and apoptotic Jurkat RNA populations, we screened the cDNA libraries using PCR amplification. We performed the amplification using primers specific for three housekeeping genes. The genes chosen were β-Actin, a highly abundant message in total RNA (3), γ-Actin, a moderately abundant message (4), and ADP Ribosylation Factor 1, a relatively low-abundance message. We also amplified the caspase-3 message, presumably of extremely low abundance but present in both apoptotic and nonapoptotic cells. Primer sequences are provided in Table 1, page 8.

All three housekeeping genes could be detected from as little as 1pg of total RNA (Figure 2), demonstrating the

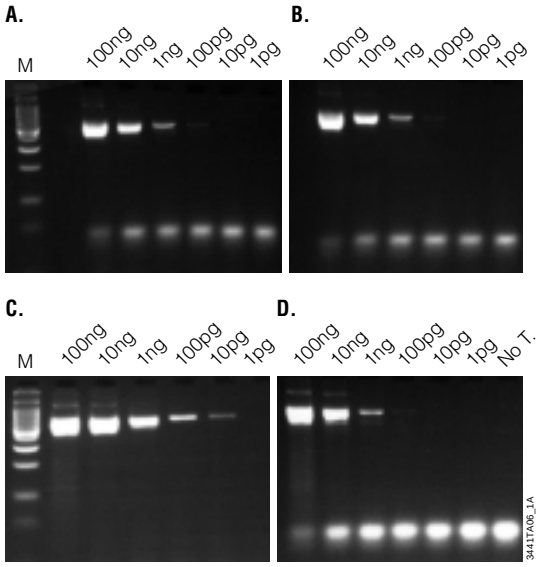

Figure 3. Sensitivity and flexibility of the ImProm-II™ Reverse Transcription System for detecting rare messages from total RNA. cDNA was synthesized with the ImProm-II™ Reverse Trascription System using the methods given at the end of this article. Titrated amounts of Jurkat cell culture total RNA (1pg–100ng) provided the template for reverse transcription. We specifically amplified the caspase-3 cDNA using uncoupled RT and PCR: Reaction I (Panel A), II (Panel C), or III (Panel B) or coupled RT-PCR (Panel D). We analyzed 10µl samples of each RT-PCR by electrophoresis on a 4% agarose gel and visualized the results by ethidium-bromide staining. Markers are the 100bp DNA Ladder.

sensitivity of the ImProm-II™ Reverse Transcription System. The caspase-3 amplicon was detectable in as little as 10pg of RNA (Figure 3C).

As expected, the data from Figure 1 show an increase in the DEVDase activity in cells induced to undergo apoptosis. The data in Figure 2 do not show any major differences between uninduced and apoptotic cells, suggesting that apoptosis does not upregulate expression of caspase-3. These data are consistent with the theory that rapid processing of the inactive proform of caspase-3 occurs during apoptosis.

Conclusion

Promega reagents allow the systematic investigation of biological processes at many levels of cellular activity. The "add, mix, measure" format of the Apo-ONE™ Homogeneous Caspase-3/7 Assay results in rapid assessment of an apoptotic event. The SV Total RNA Isolation System provides a reliable method for the isolation of high-quality RNA, and the ImProm-II™ Reverse Transcription System is a robust system for studying gene expression.

References

1. *Apo-ONE™ Homogeneous Caspase-3/7 Assay Technical Bulletin*, #TB295. Promega Corporation.
2. *ImProm-II™ Reverse Transcription System Technical Manual*, #TM236. Promega Corporation.
3. Sheuerman, R.H. and Bauer, S.R. (1993) *Meth. Enz.* **218**, 446–473.
4. Bernard, K. *et al.* (1996) *Nuc. Acids Res.* **24**, 1435–1442.
5. Adams, M.D. *et. al.* (1995) *Nature.* **377 Suppl.** 3–174.
6. Tewari, M. *et al.* (1995) *Cell* **81**, 801–809.

Detailed Protocols

***ImProm-II™ Reverse Transcription System Technical Manual*, #TM236**

(www.promega.com/tbs/tm236/tm236.pdf)

***Apo-ONE™ Homogeneous Caspase-3/7 Assay Technical Bulletin*, #TB295**

(www.promega.com/tbs/tb295/tb295.pdf)

***SV Total RNA Isolation System Technical Manual*, #TM048**

(www.promega.com/tbs/tm048/tm048.pdf)

Ordering Information

Product	Size	Cat.#
ImProm-II™ Reverse Transcription System*	100 reactions	A3800
Apo-ONE™ Homogeneous Caspase-3/7 Assay[a]	10ml	G7790
	100ml	G7791
SV Total RNA Isolation System*	50 preps	Z3100
ImProm-II™ Reverse Transcriptase*	10 reactions	A3801
	100 reactions	A3802
	500 reactions	A3803
PCR Master Mix*[b]	10 reactions	M7501
	100 reactions	M7502
	1,000 reactions	M7505
Taq DNA Polymerase*[b]	100 units	M1661

*For Laboratory Use.

[a]This product is covered by U.S. Pat. Nos. 4,557,862 and 4,640,893 and is sold for research use only. All other uses, including but not limited to use as a clinical diagnostic or therapeutic, require a separate license. Please contact Promega Corporation for details relating to obtaining a license for such other use.

[b]The PCR process is covered by patents issued and applicable in certain countries. Promega does not encourage or support the unauthorized or unlicensed use of the PCR process. Use of this product is recommended for persons that either have a license to perform PCR or are not required to obtain a license.

[c]U.S. Pat. No. 5,552,302, Australian Pat. No. 646803 and other patents.

[d]U.S. Pat. Nos. 4,966,964, 5,019,556 and 5,266,687, which claim vectors encoding a portion of human placental ribonuclease inhibitor, are exclusively licensed to Promega Corporation.

Protocol: Reaction Options for Uncoupled PCR.

1. Add Promega reagents directly to the products of the reverse transcription reaction:

	Reactions		
Component	**I Volume**	**II Volume**	**III Volume**
Reverse Transcription Reaction	20µl	20µl	20µl
Nuclease-Free Water	21.6µl	NA	13µl
PCR Master Mix*	NA	NA	15µl
Taq Reaction Buffer, 10X* (Cat.# M1901)	3.0µl	NA	NA
$MgCl_2$, 25mM*	2.4µl	NA	NA
Gene-specific primer pair	2.0µl (25µM ea.)	1.0µl (20µM ea.)	2.0µ (50µM ea.)
Promega *Taq* DNA Polymerase (Cat.# M1661; 5U/µl)	1.0µl	1.0µl	NA
Final PCR Volume	**50µl**	**22.0µl**	**50µl**

2. Place in a thermal cycler and proceed with PCR.

For a detailed protocol see Technical Manual #TM236.
N.A. Not applicable to this method.
*Volumes must be adjusted to account for dNTP and $MgCl_2$ carryover from RT reaction.

Protocol: Coupled RT-PCR.

1. Prepare Primers and Templates as described in Technical Manual #TM236.
2. For each 20µl RT-PCR volume combine the components of the ImProm-II™ Reverse Transcription System and Promega *Taq* DNA Polymerase on ice.

Nuclease-Free Water	5.9µl
ImProm-II™ 5X Reaction Buffer	4.0µl
$MgCl_2$, 25mM	1.6µl
dNTP Mix, 10mM	1.0µl
rRNasin® Ribonuclease Inhibitor[c,d]	0.5µl (20u)
ImProm-II™ Reverse Transcriptase	1.0µl
Promega *Taq* DNA Polymerase (Cat.# M1661, 5u/µl)	1.0µl
Final volume per reaction	15µl

3. Add 5µl of Target + Primer Pair combination and overlay with nuclease-free mineral oil to prevent evaporation.
4. Incubate in a thermal cycler using the following protocol.

Anneal	25°C for 5 minutes
Extend first strand	42°C for 60 minutes
Heat inactivate	95°C for 5 minutes
PCR Program	As appropriate
Final extension	As appropriate
Chill	4°C