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Dual Luciferase Assay: An Efficient Method to Study the Polyadenylation Process

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Abstract: Cleavage and polyadenylation is an obligatory step in mRNA biogenesis in eukaryotes. Poly(A) tails play an important role in mRNA turn over, transport and translation. Currently available methods for the study of polyadenylation are very cumbersome and involve radioactivity. Dual luciferase assay was found to be an efficient and equally reliable method to CAT assay and RNase protection assay. Comparison of these methods is reported in this paper.

Key words: Assay transcription, dual luciferase, polyadenylation, mRNA

Introduction

3'-end formation by post transcriptional processing is an obligatory step in the synthesis of eukaryotic mRNA. It involves a specific endonucleolytic cleavage of mRNA precursor downstream of the coding sequence, followed by template-independent addition of poly(A) tail to the upstream cleavage product. The only known exception to the above mentioned pathway is the formation of mRNAs encoding replication-dependent histones in metazoans: endonucleolytic cleavage takes place but there is no addition of poly(A) tails (Keller, 1995; Manley, 1995; Wahle and Keller, 1996). Polyadenylation is required for efficient transport of mRNA into the cytoplasm (Eckner *et al*., 1991; Huang and Carmichael, 1996). In cytoplasm, the poly(A) tails play an important role in the control of mRNA turnover and serves to stabilize it (Jacobson and Peltz, 1996). It is also well documented now that the poly(A) tail plays an essential role in the initiation of translation (Tarun and Sachs, 1996) and enhances the efficiency of translation (Jacobson and Peltz, 1996).

Reliable methods are required to study cleavage/polyadenylation process. But current information on polyadenylation rates *in vivo* is limited by the methodology available for making such measurements. One such method involves the measuring of the pulse-labelled RNA in the poly(A) + fraction of total nuclear RNA (Salditt-Georgieff *et al*., 1980). Such method is inherently limited in precision and only suitable for RNA that is abundant. Chloramphenicol acetyl transferase (CAT)assay (Gorman *et al*., 1982) and RNase protection assay (Ausubel *et al*., 1988) are other techniques those are used to study cleavage and polyadenylation process. But such methods are cumbersome, time consuming and involve radioactivity. Dual luciferase assay is a technique that utilizes luciferase gene from fire fly and Renilla and is much faster method and involves no radioactivity. It was used to study polyadenylation process in SV40 early and synthetic poly(A) sites and was found equally reliable to RNase protection and CAT assays. The results of such comparison are reported in this paper.

Materials and Methods

The plasmids required were constructed by the cloning procedures described by Maniatis *et al*. (1982). List of constructs is given in Table 1.

CAT (Chloramphenicol acetyltransferase) ASSAY: Lipofectamine (Gibco BRL) transfections were performed according to manufacture's protocols. COS-7 cells were grown in DMEM$^+$ (Dulbecco's modification of Eagle's medium containing 10% fetal calf serum and 1% antibiotics (Butler, 1991) in six-well plates at 37EC under 5% CO_2. Transfections were done at 50 to 60% confluency. All transfection experiments were performed in duplicate and with a positive CAT control in parallel. All transfection DNA included a cotransfected luciferase-expressing plasmid, pRSVluc in three times less than molar amount of the experimental plasmid. Transfections were carried out with DNA-lipofectamine mixture in DMEM only (no serum and antibiotics). DMEM solution was replaced with DMEM$^+$ five hours after transfection. The cell were washed with serum free DMEM and fed with DMEM$^+$ 20-24 hours after transfection. Cells were rinsed twice with ice cold phosphate-buffered saline (0.2 g/L KCl, 8 g/L NaCl, 0.2 g/L KH_2PO_4, 0.115 g/L Na_2HPO_4) 46-50 hours after transfection, lysed *in situ* with 900 µl of Promega's IX reporter lysis buffer by 15 minutes of incubation at room temperature, transferred to a screw-capped microfuge tube, freeze-thaved (liquid nitrogen/room temperature water), and centrifuged. 200 µl of the supernatant was removed for luciferase assay. The rest was heat-inactivated for the CAT assay at 65EC for 10 minutes, followed by a 5 minutes spin. The supernatant was stored at -60EC till analysis. 20 µl of the room temperature extract was added to 80 µl of 2X luciferase assay buffer (0.2 M K_2HPO_4 pH 7.8, 10 mM ATP, 2 mM DTT) in a 12x75 mm polystyrene test tube. The reaction was assayed in a luminometer (LUMAT LB 9501) that injects 100 µl of luciferin solution (0.1 M K_2HPO_4 pH 7.8, 1 mM DTT, 943 µM luciferin) into each reaction mixture, and then captures the light emitted for 10 seconds. All samples were assayed in duplicate and volume of extract containing equivalent amounts of luciferase activity were then assayed in duplicate for CAT activity. The procedures are modified from those developed by Gorman *et al*. (1982). 1-30 µl of heat inactivated extract (2500 RLV/µl extract) was added to 0.67 mM (final concentration) acetyl coenzyme A, 0.01 µCi [^{14}C] chloramphenicol (54 mCi/mmol), 24.8 µM unlabelled chloramphenicol, and enough 0.25M Tris pH 8.0 for a final reaction volume of 120 µl. After 20 to 60 minutes of incubation at 37EC, the reaction was stopped by ethyl acetate extraction, dried in speedvac (20 minutes with heat) and resuspended in 30 µl ethyl acetate. It was fractionated by thin layer chromatography using chloroform/methanol (95:5) as solvent. The TLC plate was dried in the hood, wrapped in saran wrap and placed in phosphorImager cassette for 12-24 hours. Quantitation was done by using a Molecular Dynamics PhosphorImager. Assay results were normalized to a positive control transfected in parallel.

Dual luciferase assay: Transfections were carried out by using Lipofectamine (Gibco BRL) as described for CAT assays. Dual

Table 1: List of plasmids constructed

pRSVcat	See Figure 1.
Caty, Caty-P$^+$	The BssHII multiple cloning site fragment of pBluescriptII SK$^+$ was cloned into the BamHI site of pRSVcat.
Caty-P	Following oligonucleotide and its complement was ligated into blunted HpaI-XbaI of Caty-P$^+$:5' CCCGGTTAACAAATAAAGC ATTTTTTTCACTGCATTCTAGTTGTGGTTTGTGGATCCCCGT.
Caty-Prev.	As for Caty-P except that the insert was placed in reverse orientation.
Caty-SPA	SPA (Levitt *et al.*, 1989) was inserted into Caty-P$^+$ cut HpaI-XbaI by blunt-sticky end ligation.
Caty-SPAα	Antisense to SPA was ligated into Caty-SPA cut SmaI-XhoI by blunt end ligation.
Caty-SPA$_{GUmut}$	Following oligonucleotide and its complement was inserted into Caty-SPA cut BglII-SpeI by sticky end ligation: 5'GATCTG TRGTGTTGGTCTAGAA.
pS*	SphI-HpaI fragment (containing CAT gene) of Caty SPA was replaced with Renilla luciferase gene [SphI-HpaI fragment from pRSVrl (Chao *et al.*, 1989)] by sticky end ligation.
Luc-P$^+$	CAT gene was replaced with Renilla luciferase gene in Caty-P$^+$ as in pS*.
pS*α	CAT gene was relaced with Renilla luciferase gene in Caty-SPAα as in pS*.
pS*$_{GUmut}$	CAT gene was relaced with Renilla luciferase gene in Caty-SPA$_{GUmut}$ as in pS*.
pS	Following oligonucleotide and its complement was placed into XhoI site of pS (Chao *et al.*, 1999) through blunt end ligation : 5'GGGACACCTGTCAGCACTAGTCAATAAAAGATCAGAGCT CTAGAGATCTGTGTGTTGGTTTTTTGTGTGTCGCCCCG
pS$_{23\alpha59}$	A 75 bp DNA frgament was inserted at the AccI site of pS by blunt-end ligation to give the sequence in Fig. 4B.
pS$_{n\alpha59}$ when n = 83, 168, 309	60 bp, 145 bp and 286 bp fragments were inserted into the HpaI site of pS$_{23\alpha59}$ to give pS$_{83\alpha59}$, pS$_{168\alpha59}$ and pS$_{309\alpha59}$ respectively (Chao *et al.*, 1999).

Table 2: CAT expression values.

Construct	Norm. cat value (avg.)	Avg. % expression	St. Dev.
pRSVcat	2.70E-06	100	
ßHpolyA	6.48E-07	95.12	13.29
Caty-P+	2.21E-06	96.09	10.28
Caty-SPA	2.54E-06	111.99	11.59
CatySPA-α	5.96E-07	17.22	5.46
CatySPA$_{GUmut}$	1.89E-06	53.75	16.72
Caty-P	1.21E-06	24.20	3.26
Caty-Prev.	3.32E-07	11.01	0.77

At least three independent transfections and six CAT assays were carried out for each construct

luciferase assay was performed by using Promega kit. 5-20 µl extract was added to 100 µl of LARII (Luciferase assay reagent II) in lumino-meter tube, mixed by pipetting 5-6 times and assayed pRSVfl in luminometer (LUMAT LB 9501). 100 µl of Stop and Glo reagent was added to the same tube, vortexed for 2 seconds and assayed again in luminometer for the experimental plasmid. All samples were assayed in duplicate and the averaged Renilla luciferase values were then normalized to the averaged firefly values. A normalized average of duplicates was additionally normalized to a positive control transfected in parallel.

RNase protection assay: RNase protection assay was performed using standard procedures (Ausubel *et al.*, 1988). Transfections were carried out by using FUGENE 6 (Boehringer Mannheim) in 100 mm plates containing 50-60% confluent cells. The DMEM+ medium was replaced 20-24 hours after transfection. The cells were rinsed twice with ice cold phosphate-buffered saline (PBS) 48-50 hours after transfection, harvested in PBS and transferred to 15 ml conical tubes. After centrifuging at 300 xg (4EC), for 5 minutes, PBS was aspirated off. The pellet was resuspended in 175 µl of RLN + DTT, incubated in ice for 5 minutes and centrifuged again at 300 xg at 4EC for 2 minutes. Cytoplasmic and nuclear RNA was isolated from supernatant and pellet respectively. For cytoplasmic RNA 600 µl of RLT (Qiagen Co.) + ß-ME (ß-mercaptoethanol) was added to the supernatant. The sample was homogenized by vortexing for 10 times or by passing it through G-20 needle for 6-8 times. RNA was then purified through Qiagen columns. 30 µl RNase free water was added to the column, centrifuged for one minute and RNA was collected in Eppendorf tube. 30 µl RNase free water was added again, centrifuged for one minute and collected in the same Eppendorf tube. Concentration was determined on spectrophotometer (Beckman). For nuclear RNA the pellet was washed once with 175 µl RLN (Qiagen Co.) and the pellet was suspended in 600 µl RLT. It was homogenized through vortexing for one minute or by passing through G-20 needle. RNA was then purified using Qiagen columns. The samples were finally resuspended in 60 µl RNase free water and quantitated on spectrophotometer.

Radiolabelled RNA probes were prepared using α-^{32}P-CTP or α-^{32}P UTP. The DNA sequence required for particular sequence was placed in inverted orientation under T_3 or T_7 promoter through cloning. Radiolabelled RNA was transcribed using T_3/T_7 promoter. The transcribed RNA probe was incubated for 60 minutes at 37EC, digested with 10U RNase free DNase I (15 min. incubation at 37EC), extracted with phenol/chloroform/isoamyl alcohol and purified through 6% polyacrylamide gel. The gel was exposed to autoradiography and the required fragment was excised from gel. RNA was eluted in 400 µl elution buffer (0.5M NH_4OAc, 1% SDS) overnight and purified using 2 µl tRNA (10 µg/µl) in ethanol (-20EC; 2 hours or more). The extracted probe was resuspended in ddH_2O and counted on scintilation counter.

All target RNA was coprecipitated with 10^6 cpm of radiolabelled probe prior to resuspension in hybridization buffer (40 mM pipes, 1 mM EDTA, 0.4M NaCl pH 6.4, 80% deionized formamide). The samples were heated to 85EC for 10 minutes and incubated overnight at 56EC. 350 µl RNase digestion buffer (5 mM EDTA, 300 mM NaCl, 10 mM Tris pH 7.5) containing 1 µl T_1 RNase was added to digest the unprotected RNA and probe and incubated at 30 EC for 30 minutes. The reaction was stopped by adding 20 µl of 10% (wt/vol) SDS and 2.5 µl of 2.0 mg/ml proteinase K by incubating at 37EC for 15 minutes. Extraction was carried out once with 400 µl phenol/

Table 3: Comparison of dual luciferase assay (DLA) and CAT assay

Construct CAT name	Avg % expression (CAT assay)	St. Dev.	Construct DL Assay name	Avg% expression (luciferase)	St.Dev.
Caty-SPA	111.99	pS*	100		
Caty-P^+	96.09	10.28	Luc-P^+	94.32	12.85
CatySPA-α	17.22	5.46	pS*α	26.72	4.32
$CatySPA_{GUmut}$	53.75	16.72	pS^*_{GUmut}	62.85	8.56

chloroform, removing the aqueous phase to a clean microcentrifuge tube containing 1 µl of 10 mg/ml tRNA. The extracted sample was ethanol precipitated, washed with 70% ethanol, dried and resuspended in 7 M urea dye. It was incubated at 85EC for 5 minutes and loaded on 6% denaturing polyacrylamide/7 M urea gel. The gel was run for 3-5 hours at 500 V, dried and placed on phosphorImager screen overnight. The bands were quantified using Image Quant software.

Results and Discussion

CAT Assay Versus Dual Luciferase Assay

CAT assay: CAT is a bacterial gene encoding chloramphenicol acetyl transferase enzyme. It acetylates ^{14}C-labelled chloramphenicol using acetyl CoA. The acetylated and free chloramphenicol are separated using thin layer chromatography (TLC). The plates are exposed to phosphor screen on phosphorImager and quantified using Image Quant. CAT gene is found better in eukaryotic gene expression studies as it is not present inherently in the eukaryoties. Hence the expression is solely from the transfected DNA.

Figure 1 shows the basic construct, pRSVcat (Gorman *et al.*, 1982). It has CAT gene and a small intron with RSV promoter. HpaI-BamHI region contains SV40 early poly(A) site. This site contains two tandem poly(A) sites; downstream being the major one i.e. stronger as compared to the upstream one (Connelly and Manley, 1988). Caty was derived from pRSVcat by putting multiple cloning site downstream of BamHI in pRSVcat. Caty-P^+ has complete SV40 poly(A) site (with two poly(A) sites). Multiple cloning site was used for further DNA insertions. The CAT assay results for Caty-P^+ were comparable to pRSVcat (Table 2, Fig. 2). The CAT assay results were normalized to luciferase activity. Luciferase gene was inserted in another plasmid and co-transfected with the experimental plasmid containing CAT gene. Luciferase gene is obtained from fire fly and has same advantages as of CAT gene. Average percent expression was obtained by comparing the normalized CAT values to the positive control transfected in parallel with all experimental plasmids. Positive control was being pRSVcat.

In order to study polyadenylation process precisely, it was necessary to use a poly(A) site having minimal sequence required for polyadenylation. Therefore, Caty-P was made by deleting out upstream poly(A) site from SV40 early poly(A) site; 198 bp long fragment containing SV40 early poly(A) site was replaced with short P containing only the downstream poly(A) site. The CAT activity reduced drastically for Caty-P i.e. 24.2% as compared to pRSVcat (Fig. 3, Table 2). Therefore, Caty-P could not be used to study the polyadenylation reaction. It was, therefore, necessary to select another poly(A) site having minimal polyadenylation elements and maximal polyadenylation reaction for further studies. Synthetic poly(A) site (SPA) of Levitt *et al*. (1989) was selected for this purpose (Fig. 4). It was synthesized on oligosynthesizer and inserted at HpaI-XbaI site in Caty. The CAT activity for Caty-SPA (Fig. 2) was found comparable to pRSVcat poly(A) site and ßH poly(A) site. ßH poly(A) site was derived from rabbit globin gene (Pribyl and Martinson, 1988). On the basis of these results, SPA was selected for use in most of the further studies.

Caty-Prev. was made by placing "P" in reverse orientation in Caty. Lower CAT activity in Caty-Prev. shows that the activity in Caty-P was due to SV40 early poly(A) site. The residual activity (11.01%) might be due to cryptic poly(A) sites present downstream of the authentic poly(A) site. Antisense constructs (Caty-SPAα) were constructed by putting inverted copy of SPA, 23 bp downstream of the poly(A) site (Fig. 4). The expression was inhibited due to cis-antisense inhibition (Chao *et al.*, 1999). In order to confirm that polyadenylation was taking place at the synthetic poly(A) site and not at any other cryptic poly(A) site, mutations were in the GT rich region of Caty-SPA to give Caty-SPA_{GUmut}. It was shown earlier (Connelly and Manley, 1988) that a poly(A) site becomes weaker if some of the Gs or Ts were removed from the poly(A) site. Construct used in the present studies had following basis removed: TGTGTGTTTTT. It was transfected in COS cells and assayed for CAT expression. As shown in Table 2, Caty-SPA_{GUmut} had much lower CAT activity (53.75"16.72%) as compared to the positive control. This suggests that the CAT activity in Caty-SPA was due to the authentic poly(A) site and GT rich region is essential for efficient polyadenylation.

Dual Luciferase Assay: In DLA, CAT gene was replaced with coelentrate (Renilla) luciferase gene in experimental plasmids. The experimental plasmid was cotransfected with the plasmid containing fire fly luciferase gene. The reaction is performed in the single tube. Table 3 shows comparison of DLA and CAT assay. It was found that both assays gave comparable results. Dual luciferase assay has showed good reproducibility with very low standard deviation. CAT assay values were normalized to pRSVcat as positive control. Whereas for dual luciferase assay, pS* was used as positive control. The trend in both set of constructs was same. The GT mutant construct (pS^*_{GUmut}) with luciferase gene also showed significant decrease in polyadenylation activity as in Caty SPA_{GUmut}. Similar is the case with pS*α compared to Caty-SPAα. The constructs with pS* had luciferase gene in the plasmids containing SPA site whereas Luc-p^+ had SV40 early (complete) poly(A) site. Both had very close expression values. Present results also suggest that the assay is independent of the type of poly(A) site and, therefore, can be used on any of the poly(A) site.

RNase Protection Assay versus Dual Luciferase Assay: In RNase protection assay radiolabelled probe, covering the region upstream and downstream of poly(A) site, is made. It is hybridized with the target RNA isolated from cell after transfection. The hybridized product is digested with RNase that digests only single stranded RNA. The undigested fragments are quantified on phosphorImager after running on denaturing gel. Cleavage and polyadenylation takes place at poly(A) site in between hexamer and GU/U rich element (Connelly and Manley, 1988). Ratio of cleaved to read through RNA gives cleavage/polyadenylation percentage. Rnase

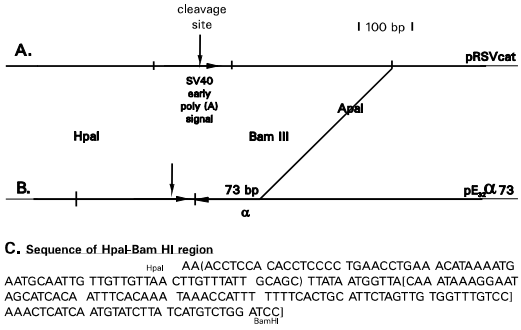


Fig. 1: Construction of pRSVcat. (A) The 3' end of the CAT transcription unit of pRSVcat. (B) The 3' end of the CAT transcription unit of $pE_{34}\alpha_{73}$ (construct irrelavant to present study). (C) Sequence of the 3' end of pRSVcat. The regions targeted by antisense in the $pE\alpha_{73}$ series of constructs are enclosed in brackets. The sequence within the brackets actually encodes two interdigitated poly(A) signals. The upstream and downstream motifs for the dominant signal are shown in bold and are used exclusively *in vivo* to direct cleavage as shown by the arrow

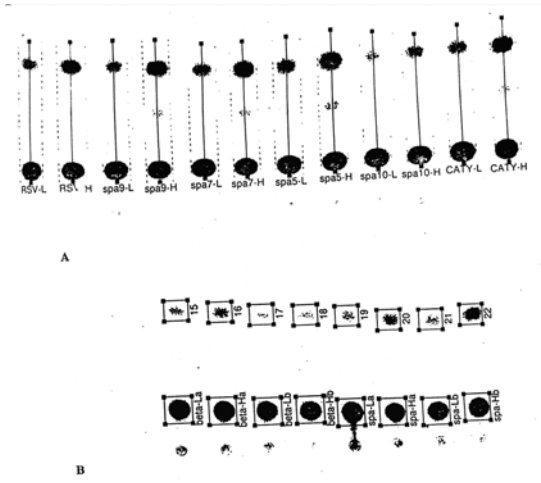


Fig. 2: PhosphorImager Scan for CAT assay.
A. pRSVcat (RSV), CATY-P^+ (Caty) and CATY-SPA (SPA 9, 5, 7 and 10 are different maxipreps of Caty-SPA). L and H are low and high conc. of the reaction, respectively.
B. Comparison of ßH poly(A) and Caty-SPA

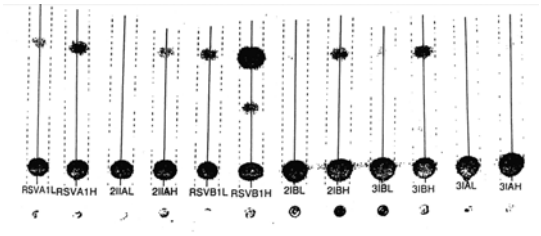


Fig. 3: CAT assay scan for pRSVcat and Caty-P. 2A, 2B, 3A and 3B are different maxipreps. of Caty-P (containing single SV40E poly(A) site)

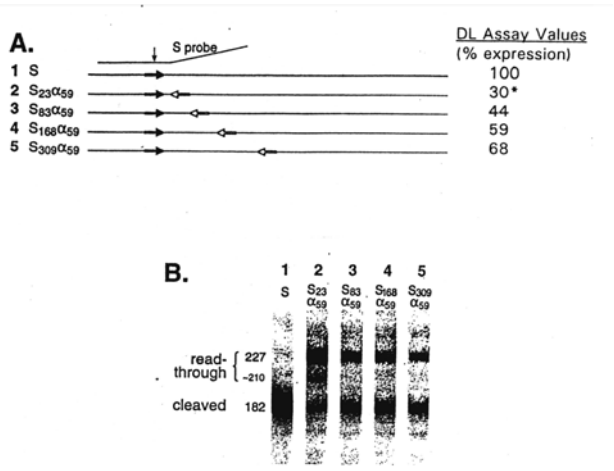
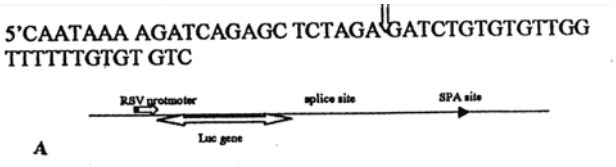


Fig. 4: A. Synthetic poly(A) (SPA). SPA was placed in Caty using HpaI/XbaI (blunted) digestion
B. Sequence of 3' end of $S_{23}\alpha_{59}$ with antisense sequence that was placed downstream of HpaI site in different SPA containing constructs



Sequence of sense-antisense region in S23
tgtcaGCAT AGTCAATAAA AGATCAGAGC TCTAGACATC TGTGTGTTGG TTTTTTGTGT GTCGcccgga tctcgaggtc ggHpaIttaacCGA CACACAAAAA ACCAACACAC AGATCTCTAG AGCTCTGATC TTTTATTGAC TAGTGCcctg tcaccccgat

Fig. 5: (A) Diagrammatic representation, drawn to scale, of the plasmids and probes used for Part B
(B) RNase protection assays of nuclear RNA. The S probe was a T3 RNA polymerase transcript of a DraI-digested plasmid obtained by inserting the PvuII fragment of pBluescript SK + into the HpaI site of $pS_{23}\alpha_{59}$. The RNA lengths given in the figure refer to cleavage at susceptible Gs. The probe has a variable tendency to be cleaved at an internal position in the readthrough portion

protection assay was performed on the constructs with SPA as polyadenylation site. pS had 100% cleavage whereas other constructs in the series had cleavage percentage as 28, 47, 56 and 71 in $S_{23\alpha59}$, $S_{83\alpha59}$, $S_{168\alpha59}$, and $S_{309\alpha59}$, respectively (Fig. 5).

Same constructs were subjected to dual luciferase assay. The percent expression values (Fig. 5) were found similar to those obtained through RNase protection. It shows validity of the

dual luciferase assay that can replace cumbersome procedure of RNase protection assay for the study of polyadenylation process. However, RNase protection assay is necessary to see the cleaved and uncleaved fragments thereby confirming the idea that at which site polyadenylation process is taking place. CAT assay and RNase protection assay can be replaced with dual luciferase assay for polyadenylation studies. DLA has advantage over the other two that no radioactivity is involved in it and is faster and equally reliable to both of the assays.

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