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A Comparative Study of T7 RNA Polymerase Quality

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Many vendors supply bacteriophage RNA polymerases for in vitro *transcription applications; however, the unit activity assays used by these vendors vary widely. In order to test the consistency and reproducibility of the results obtained from different enzyme sources, we compared T7 RNA polymerases from six leading vendors. We measured unit activity and performed standard quality assurance tests. In this article, we present the results of our comparisons.*

Introduction

Several vendors provide bacteriophage (phage) RNA polymerases for *in vitro* transcription. Quality assurance specifications and assays for these enzymes differ among vendors, making the results difficult to compare. Even unit definitions may vary since each vendor uses different assay components and concentrations, a different DNA template, and measures incorporation of single or multiple nucleotides. In particular, DNA templates used by different vendors consist of either linearized plasmid DNA (to produce run-off transcripts), supercoiled plasmid DNA or phage DNA. This variation in choice of template introduces a corresponding variability in the unit activity of the enzyme. Template factors that can affect activity include DNA form (linear or supercoiled), the number of promoters per template, the size of the transcript, the presence of terminator sequences and promoter strength. These differences in unit activity can interfere with the reliability and consistency of experiments when phage RNA polymerases from different vendors are used.

Promega is committed to providing high-quality enzymes that ensure reproducible and consistent results. Recently, we evaluated the activity assays used for our phage RNA polymerases for consistency. In addition, we compared Promega's T7 RNA Polymerase with enzymes from other vendors. In particular, we wished to evaluate how the unit activity compared and how the enzymes performed in the same quality assurance assays.

Unit definition assays

Unit activity assays for phage RNA polymerases have evolved over time. For example, early workers and suppliers of RNA polymerases used the specific phage DNA as the template for activity assays (1-3). However, it is now common practice to use other DNA templates. Recently, we examined Promega's phage RNA polymerase unit activity assays for consistency, and we consequently modified the unit definition. While optimizing the assay, we kept the amount of activity per unit volume the same to ensure that no change in protocols would be necessary for the end user. Since the activity of enzyme remains the same, one new unit equals one old unit. The new unit definitions are as follows for each phage RNA polymerase:

For T7 and SP6 RNA Polymerases

One unit is defined as the amount of enzyme required to catalyze the incorporation of 5nmol of CTP into acid-insoluble product in 60 minutes at 37°C in a total volume of 100µl. The reaction conditions are: 40mM Tris-HCl (pH 7.9), 6mM $MgCl_2$, 10mM DTT, 10mM NaCl, 2mM spermidine, 0.05% Tween®-20 (ICI Americas, Inc.), 0.5mM each of ATP, GTP, CTP and UTP, 0.5µCi [^{3}H]CTP and 2µg of supercoiled pGEM®*-5Zf(+) Vector DNA.

**U.S. Pat. No. 4,766,072 has been issued to Promega Corporation for transcription vectors having two different bacteriophage RNA polymerase promoter sequences separated by a series of unique restriction sites into which foreign DNA can be inserted.*

For T3 RNA Polymerase

One unit is defined as the amount of enzyme required to catalyze the incorporation of 5nmol of CTP into acid-insoluble product in 60 minutes at 37°C in a total volume of 100µl. The reaction conditions are: 40mM Tris-HCl (pH 7.9), 6mM $MgCl_2$, 10mM DTT, 10mM NaCl, 2mM spermidine, 0.05% Tween®-20, 0.5mM each of ATP, GTP, CTP and UTP, 0.5µCi [^{3}H]CTP and 2µg of supercoiled pSP6/T3 Vector DNA.

Quality assurance assays

To assess the quality of Promega's T7 RNA Polymerase, we compared it to enzyme from six other leading vendors, using Promega's activity assay and other quality assurance assays. Since different vendors use different activity assays and unit definitions, the activity assays were performed using Promega's standard assay so that the unit concentration obtained for each vendor could be directly compared. These measured units were subsequently used as a reference point to ensure that the same amount of T7 RNA polymerase was added to each of the quality assurance assays. The measured units were also compared with the units per microliter given on the

label by each vendor, to compare the unit definition of each vendor with that of Promega. In this comparison, we assumed that the unit concentration stated by each vendor was accurate.

Enzyme activity assay

For the activity assays, we prepared serial dilutions of each enzyme in Promega's storage buffer to produce T7 RNA polymerase concentrations in the linear range of the assay. Four microliters of diluted enzyme was added to a 100µl reaction mixture containing 2µg of supercoiled pGEM®-5Zf(+) Vector DNA, 40mM Tris-HCl (pH 7.9), 6mM $MgCl_2$, 2mM spermidine, 10mM NaCl, 10mM DTT, 0.5mM each of ATP, CTP, GTP and UTP, 0.5µCi [^{3}H]CTP (15-25Ci/mmol) and 0.05% Tween®-20. The reaction was incubated at 37° C for 10 minutes. Each sample was TCA-precipitated and filtered through a Whatman® GF/A filter (Whatman Paper Company, Ltd.) to determine the percent incorporation (4). The number of units per microliter was calculated using the unit definition given above from multiple replicate experiments. Finally, "equivalent standard units" were calculated for each vendor (measured units/µl ÷ label units/µl).

The results of these experiments are provided in Table 1. The equivalent standard units illustrate that one unit of activity from a given vendor may not correspond to one unit of activity from another vendor. This discrepancy could cause variability in the results obtained from following the same protocol, but using enzymes from different vendors.

Table 1. Unit Activity Assay Comparison.

Vendor	Equivalent Standard Units*
Promega	1.0
A	0.6
B	1.2
C	0.5
D	0.9
E	0.3
F	0.2

*For vendors with multiple enzyme sizes, the equivalent standard units were averaged.

Protein purity assay

We analyzed T7 RNA polymerase preparations from different vendors by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) to determine the enzyme's physical purity. Contaminating proteins are undesirable since they could affect the performance of *in vitro* transcription and the ability to produce full-length transcripts if the contaminant is an endonuclease or exonuclease. Three protein amounts (5, 25 and 50 units/lane) were loaded on 4-20% Tris-glycine gels. Gels were stained with Coomassie® blue (Imperial Chemical Industries), destained and the percent contaminating protein was estimated visually. Promega's quality assurance specifications for this assay require that the protein should be a single band, >=90% pure at 5, 25 and 50 units.

No contaminating proteins were seen on the gels for the T7 RNA polymerase from Promega and all other vendors except for Vendor F. Vendor D's preparation contains BSA as part of the storage buffer; this was detected on the gels but was not considered a contaminant. For Vendor F, a single contaminating band with a molecular weight of approximately 80,000kDa was detected. This band comprised approximately 10% of the total protein.

Endonuclease/nickase assay

Promega's endonuclease assay was performed to detect endonucleases or nickases that could cleave the DNA template and lead to generation of incomplete transcripts. In this assay, increasing amounts of T7 RNA polymerase (100, 200 and 300 units) were incubated with 1µg of supercoiled pGEM®-5Zf(+) Vector DNA in Transcription 1X Buffer [40mM Tris-HCl (pH 7.9), 6mM $MgCl_2$, 2mM spermidine, 10mM NaCl] for 1 hour at 37°C. DNA loading buffer was added to the reactions and the samples were analyzed on an agarose gel, followed by visualization with ethidium bromide staining. The amount of DNA remaining in Form I (supercoiled) was estimated visually. Promega's quality assurance specifications for these assay conditions are >90% Form I (supercoiled) plasmid DNA at 100 units of T7 RNA Polymerase. The 200 unit and 300 unit treatments provide a more rigorous test of endonuclease contamination.

The T7 RNA polymerase from each vendor had >90% Form I plasmid DNA remaining at all tested amounts of enzyme. Thus, all enzymes met and exceeded the specifications for endonuclease/nickase contamination.

DNase and RNase assays

Promega's standard DNase and RNase assays were performed to detect contaminating DNases that could degrade the DNA template and RNases that could degrade the RNA transcripts. The DNase and RNase assays are identical except that different substrates are used -- 50ng of ^{3}H-DNA or ^{3}H-RNA, respectively. For these assays, the appropriate volume of enzyme and storage buffer (a total volume of 30µl) was added to a 100µl reaction mixture such that the final amount of enzyme was either 100, 200 or 300 units. Two different

reaction buffers were used. The first set of reactions contained transcription buffer minus spermidine [40mM Tris-HCl (pH 7.9), 6mM $MgCl_2$, 10mM NaCl]. The second set of reactions was performed in MULTI-CORE™ 1X Buffer [25mM Tris-acetate (pH 7.8), 100mM potassium acetate, 10mM magnesium acetate, 1mM DTT] since this buffer is useful for detecting some nucleases. The reactions were incubated at 37°C for 1 hour. The percent release of the radioactive nucleotides was monitored by scintillation counting of TCA-soluble material. Promega's quality assurance specifications for these assay conditions require <1% release with 100 units of T7 RNA Polymerase. The 200 and 300 unit treatments provide a more rigorous test of contamination.

The T7 RNA polymerase from all vendors exhibited <1% release for the DNase assay at the unit concentrations tested. Thus, all enzymes met and exceeded the specifications for DNase contamination.

The T7 RNA polymerases from Promega and Vendors A, B and D met and exceeded the quality assurance specification for RNase contamination, with <1% release of radioactivity (Table 2). However, RNase contamination was detected in the T7 RNA polymerase preparations from Vendors C, E and F (Table 2). For Vendors C and F, the enzymes did not meet Promega's quality assurance specifications for RNase contamination since there was <1% release even at the 100 unit level. For Vendor E, the enzyme met Promega's quality assurance specifications, but contamination was evident at higher enzyme concentrations (Table 2). For Vendors C, E and F, large volumes of polymerase were added to the assay since the unit activity from these vendors is much lower compared to the other vendors. It is possible that these vendors may not assay this level of T7 RNA polymerase in their quality assurance tests.

Table 2. RNase Assay Results (Percent Release).

Units Tested	Promega	Vendor A	Vendor B	Vendor C	Vendor D	Vendor E	Vendor F
Transcription buffer minus spermidine							
100 units	<1%	<1%	<1%	4%	<1%	<1%	1.6%
200 units	<1%	<1%	<1%	7%	<1%	1.6%	4%
300 units	<1%	<1%	<1%	7%	<1%	N.D.	N.D.
MULTI-CORE™ Buffer							
100 units	<1%	<1%	<1%	6%	<1%	<1%	1.7%
200 units	<1%	<1%	<1%	8%	<1%	<1%	4%
300 units	<1%	<1%	<1%	14%	<1%	1.5%	N.D.

N.D., not determined.

Native agarose gel analysis of transcripts

We analyzed RNA from transcription reactions by native agarose gel electrophoresis to detect incomplete transcripts. This problem can occur due to RNA degradation, premature termination of transcription or degradation of the DNA template by endonucleases or DNases. The assay also confirms that the transcript is the correct size and that the polymerase has the correct specificity for the promoter. For this assay, each enzyme was serially diluted in Promega's storage buffer to enable two different amounts (5 and 10 units) of enzyme to be assayed. Four microliters of diluted enzyme were added to a 20µl reaction mixture containing 1.0µg of pGEM® Express Positive Control DNA, 40mM Tris-HCl (pH 7.9), 6mM $MgCl_2$, 2mM spermidine, 10mM NaCl, 10mM DTT, and 0.5mM each of ATP, CTP, GTP and UTP. The reaction was incubated at 37°C for 1 hour. The transcripts were denatured at 65°C for 10 minutes in RNA sample buffer and RNA loading buffer (4), analyzed on a 1% TAE agarose gel, and visualized by ethidium bromide staining. The transcript size and amount of degradation was estimated visually. The quality assurance specifications for this assay are to obtain transcripts of correct size, intact and with no degradation at 5 and 10 units of T7 RNA Polymerase.

For all vendors the transcripts generated in this experiment were the correct size, intact and showed no degradation. Thus, all enzymes met the specifications of this assay. No degradation of RNA was seen with the T7 RNA polymerases of Vendors C, E and F even though RNases were detected in the RNase assay. This may be due to the fact that fewer units of polymerase were used for the transcription reactions in the agarose gel assay as compared to the RNase assay.

Transcription assay

Performing transcription reactions with increasing levels of enzyme at limiting NTP concentrations provides a method to assess enzyme activity under conditions for radioactive labeling, where the concentration of one NTP is limiting. Poor incorporation of the radiolabeled NTP would result in probes with low specific activity. For this assay, each enzyme was serially diluted in Promega's storage buffer such that reactions contained 20, 10, 5, 2 and 1 unit of T7 RNA polymerase. For each reaction, 2µl of diluted enzyme was added to a 50µl reaction mixture containing 1µg of supercoiled pGEM®-5Zf(+) Vector DNA, 40mM Tris-HCl (pH 7.9), 6mM $MgCl_2$, 2mM spermidine, 10mM NaCl, 10mM DTT, 0.5mM each of ATP, GTP and UTP, and 12.0µM [^{3}H]CTP (15-25Ci/mmol). The reaction was incubated at 37°C for 1 hour. Each sample was TCA-precipitated to determine percent incorporation (4). Promega's quality assurance specifications require that >70% incorporation is obtained with 5 units of T7 RNA Polymerase under these conditions.

The T7 RNA polymerase from each vendor gave >90% incorporation when 5 units of enzyme were used, all well above the 70% incorporation level required by the specification. In the reactions with 2 units of polymerase, all samples gave >80% incorporation. In the reactions with 1 unit of polymerase, all samples except that from Vendor F gave >80% incorporation (enzyme from Vendor F gave 73% incorporation). Thus, all enzymes met and exceeded the specifications for incorporation of radiolabeled NTP under limiting NTP concentrations.

Summary

The results of unit activity assays demonstrate that T7 RNA polymerase activity from different vendors can vary significantly. This can lead to inconsistent results if enzymes from two different vendors are used in the same protocol. A summary of the data obtained from several quality assurance assays (Table 3) shows that comparable results can generally be obtained if these differences in unit activity are taken into consideration. However, a notable exception was observed in the case of RNase activity, where enzyme from two vendors did not meet Promega's quality assurance specifications. These results demonstrate the quality of Promega's T7 RNA Polymerase and ensure that users will obtain reproducible and reliable results with this enzyme.

Table 3. Summary of Results of Quality Assurance Assays.

Vendor	Protein Purity	Endonuclease	DNase	RNase	Intact Transcripts	Percent Incorporation
Promega	++	++	++	++	+	++
A	++	++	++	++	+	++
B	++	++	++	++	+	++
C	++	++	++	–	+	++
D	++	++	++	++	+	++
E	++	++	++	+	+	++
F	–	++	++	–	+	++

++ Met and exceeded Promega's quality assurance specifications.
+ Met Promega's quality assurance specifications.
– Did not meet Promega's quality assurance specifications.

References

1. Chamberlin, M., McGrath, J. and Waskell, L. (1970) *Nature* **228**, 227.
2. Chamberlin, M. and Ring, J. (1973) *J. Biol. Chem*. **248**, 2235.
3. Butler, E.T. and Chamberlin, M.J. (1982) *J. Biol. Chem*. **257**, 5772.
4. *Protocols and Applications Guide, Third Edition* (1996) Promega Corporation.

Ordering Information

Product	Size	Cat.#
SP6 RNA Polymerase	1,000u	P1085
	5,000u	P1081
	2,500u (HC)	P4084
T7 RNA Polymerase	1,000u	P2075
	5,000u	P2077
	10,000u (HC)	P4074
T3 RNA Polymerase	1,000u	P2083
	2,500u (HC)	P4024

HC = High Concentration

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