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Liquid Chromatography-Mass Spectrometry Analysis of DNA Polymerase Reaction Products

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Abstract

This unit describes experimental and analytical procedures for characterizing the efficiency and fidelity of translesion DNA synthesis across various DNA damages by DNA polymerases in vitro. This procedure utilizes primer extension assays followed by LC-MS and LC-MS/MS analysis of the extension products. Detailed explanations for the analysis of the LC-MS/MS data for deciphering the nucleotide sequences of the DNA fragments are also presented. This approach provides a significant improvement over conventional methods, as it allows detection of misincorporation, as well as frameshift products.

Keywords

DNA polymerase; translesion synthesis; sequencing; LC-MS

This unit contains procedures for LC-MS-based approaches for the sequence analysis of DNA primer extension products that result from DNA synthesis (by DNA polymerases) across various DNA lesions (i.e., modified DNA bases or sugars, e.g., *O*⁶-methyl guanine, *O*⁶-benzyl guanine, 8-oxo-7,8-dihydroguanine). A complete procedure for the primer extension assays across a DNA lesion by DNA polymerase (Fig. 7.16.1), followed by analysis of the extension products using ESI-LC-tandem MS (*UNIT 10.2*) is described here. We also present explanations for the analysis of the LC-MS/MS data for deciphering the nucleotide sequences of the DNA fragments.

Strategic Planning

Lesion-containing oligonucleotide template

The oligonucleotide template containing the lesion is either obtained commercially or has to be synthesized in the laboratory. Typically, the oligonucleotide containing the lesion is synthesized using the standard phosphoramidite chemistry in an automated DNA synthesizer (*UNIT 3.5*). However, depending on the lesion, special approaches may be required (Guengerich, 2006).

Design of the primer

The sequence of the primer should be complementary to the template and contain one or more 2'-deoxyuridine (dU) residues in place of 2'-deoxythymidine (dT), with the exception of the 3' terminal base (Fig. 7.16.1). The first 2'-dT from the 3' terminal of the deoxyoligonucleotide primer is almost always replaced with dU. If necessary, dU residues can be used to replace nucleosides other than dT (creating mismatches) if far enough into the double-stranded region and not so frequent as to disrupt overall duplex stability. The introduction of dU in the primer is a key feature of this method, which allows the extended primer to be cleaved at the site of the uracil using uracil DNA glycosylase (UDG), followed

by hot piperidine, into short pieces of DNA that are small enough for analysis by mass spectrometry.

The length of the primer can vary depending on the type of experiment one wishes to perform; however, the T_m of the primer:template complex should not be $<40^{\circ}\text{C}$.

Primer extension assays

Primer extension assays are initially performed with a 5'- ^{32}P end-labeled deoxyoligonucleotide primer and the products are analyzed using polyacrylamide gel electrophoresis, e.g., see (Zang et al., 2005). This initial assay is a convenient way to understand the expected length and complexity of the extension products. It also helps in determining the set of conditions necessary to get a reasonably good yield of the extension reaction for sequence analysis.

Materials

DNA template (see Strategic Planning)
Primer (see Strategic Planning)
Tris Cl buffer, pH 7.7 (see recipe)
DNA polymerase
Dithiothreitol (DTT)
NaCl
 MgCl_2
Bovine serum albumin (BSA; Sigma)
HPLC-grade water (Fisher Scientific)
2'-Deoxyribonucleotide triphosphates (dNTP; Sigma)
Uracil-DNA glycosylase (UDG; Sigma)
Piperidine (Sigma-Aldrich)
Acetonitrile (CH_3CN), HPLC grade (Fisher Scientific)
Ammonium acetate ($\text{NH}_4\text{CH}_3\text{CO}_2$) (Fisher Scientific)
2'-Deoxyoligonucleotide (Midland)
Heating block
 37°C incubator
Bio-Spin 6 spin column (Bio-Rad)
Swinging-bucket centrifuge
Centrifugal lyophilizer device [e.g., Centrivac (Labconco) or SpeedVac]
Acquity ultraperformance liquid chromatography (UPLC) system (Waters Associates)
Finnigan LTQ mass spectrometer (Thermo Fisher)
Acquity BEH octadecylsilane (C18) UPLC column ($1.7\text{ }\mu\text{m}$ particle size, $1.0\text{ mm} \times 100\text{ mm}$; Waters Associates)
Oligo Composition Calculator v1.2 software (<http://library.med.utah.edu/masspec/>)

Mongo Oligo Mass Calculator v2.0 (<http://library.med.utah.edu/masspec/mongo.htm>)

Anneal the primer-template complex

1. Mix equimolar amounts of the DNA template containing the adduct and the primer containing dU in 25 to 100 mM Tris·Cl buffer. Adjust the final volume to 200 μ L.
2. Heat the DNA solution at 90°C for 5 min in a heating block and then allow to slowly cool to room temperature.

Full-length extension of the primer by DNA polymerase

3. Mix 0.5 to 4 nmol DNA (primer-template) and 50 to 500 pmol DNA polymerase in 50 mM Tris·Cl buffer (pH 7.7) containing 2 mM DTT, 50 mM NaCl, 5 mM MgCl₂, and 100 μ g of bovine serum albumin.

The final volume was adjusted to 100 μ L with distilled water.

4. Initiate the reaction by adding 1 mM each of four dNTPs and incubate for 4 hr at 37°C.

The temperature may be raised for some thermostable DNA polymerases.

5. Equilibrate a Bio-Spin 6 spin column with 500 μ L water. For equilibration, first centrifuge the spin column in a swinging-bucket centrifuge 10 min at 2400 $\times g$, 25°C, to remove the solution in which the column was supplied. Add 500 μ L distilled water and centrifuge again in a swinging-bucket rotor in a centrifuge 10 min at 2400 $\times g$, 25°C. Repeat this process twice. Discard the filtrate and use the column in the next step.

6. Following the incubation, load the reaction mixture onto the equilibrated spin column and centrifuge for 20 min at 2400 $\times g$, 25°C. Collect the filtrate for the next step.

This process removes the excess dNTPs and terminates the reaction.

The filtrate contains the DNA.

Perform UDG treatment and piperidine workup to generate small pieces of DNA (Fig. 7.16.1)

7. To the above filtrate add 50 mM Tris·Cl buffer (pH 7.7), 2 mM DTT, and 6 U UDG.

The final volume is made up to 200 μ L with distilled H₂O.

UDG specifically removes dU from DNA, resulting in an abasic site.

8. Incubate the reaction mixture for 5 hr at 37°C.
9. Add 10 μ L of 10 M piperidine and heat at 95°C for 30 to 60 min.

The treatment with hot piperidine will result in the cleavage of the DNA at abasic sites created by the UDG treatment.

10. Evaporate the reaction mixture to dryness in a centrifugal lyophilizer device (CentriVac or SpeedVac).
11. Resuspend the residue in 100 μ L distilled H₂O and evaporate to dryness in a centrifugal lyophilizer device. Repeat this step twice.

12. Finally resuspend the dried residue in 200 μ L distilled H₂O for LC-MS analysis.

Prepare the instruments and columns for LC-MS analysis

13. For LC-MS and LC-MS/MS analysis of the extension products, use an Acquity UPLC system connected to a Finnigan LTQ mass spectrometer, or an equivalent ion-trap instrument. Operate the MS in the electrospray ionization (ESI) negative ion mode.
14. Use an Acquity BEH octadecylsilane (C18) UPLC column (1.7 μ m particle size, 1.0 mm $\times$ 100 mm) for liquid chromatography (LC).

Conditions for LC

15. Mobile phases for LC:
buffer A: 98% H₂O, 2% CH₃CN (v/v), and 10 mM NH₄CH₃CO₂
buffer B: 5% H₂O, 95% CH₃CN (v/v), and 10 mM NH₄CH₃CO₂.
16. Prepare the gradient program for LC according to Table 7.16.1.
17. Maintain the column temperature at 50°C.

Detect and identify the extension products

18. Tune the LTQ mass spectrometer using a 2'-deoxyoligonucleotide of appropriate (similar) length and sequence. ESI conditions generally used are: source voltage 4 kV, source current 100 μ A, auxiliary gas flow rate setting 20, sweep gas flow rate setting 5, sheath gas flow setting 34, capillary voltage -49 V, capillary temperature 350°C, tube lens voltage -90 V. MS/MS conditions were as follows: normalized collision energy 35%, activation Q 0.250, and activation time 30 ms.
19. Equilibrate the Acquity C18 column with 100% mobile phase A at 150 μ L/min for 30 min.
20. Infuse 10 μ L of each sample with an autosampler system.
21. Acquire ion spectra over the range of m/z 300 to 2000.
22. Analyze the collected LC-MS data for peaks that may correspond to a 2'-deoxyoligonucleotide containing a 5'-phosphate.

Generally, peaks corresponding to 2'-deoxyoligonucleotides will appear as multiply-charged species in the MS spectrum.

23. Deconvolute the multiple charged peaks either manually or using available software to obtain the molecular mass of the potential DNA fragments.
24. Use the Web-based, freely available software Oligo Composition Calculator v1.2 to determine the base composition of the multiply charged species (Ni et al., 1996).

IMPORTANT NOTE: *If the mass of the multiply charged species does not correspond to DNA, the software will not provide any results or—if it does—the composition may not seem reasonable based on the complementary template sequence.*

25. Identify all the peaks in the chromatogram that correspond to DNA using the Oligo Composition Calculator.

Calculate CID of detected peaks for sequence analysis

26. Inject another 10 μ L of sample and perform LC-MS/MS (collision-induced dissociation, CID) on the identified peaks (Fig. 7.16.2).
27. Acquire product ion spectra over the range m/z 400 to 2000.
28. Use the Web-based freely available software Mongo Oligo Mass Calculator v2.0 to calculate the theoretical a–B and w ions of all possible DNA sequences, based on the Oligo composition data obtained from LC-MS analysis (Ni et al., 1996). (An example is shown in Tables 7.16.2 and 7.16.3.)
29. Compare the calculated and observed a–B and w ion spectra.

The DNA sequence whose calculated and experimental ion spectra match is the sequence of that extension product. Mixtures are expected in many cases, especially when working with incorporation opposite and beyond modified residues (Choi et al, 2006).
30. Calculate the relative yield of the various identified DNA extension products based on their respective peak areas in the LC-MS chromatogram.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, SEE APPENDIX 2A; for suppliers, SEE SUPPLIERS APPENDIX.

Tris-Cl buffer, pH 7.7

To prepare 100 mL of 1 M Tris-Cl buffer, pH 7.7, dissolve 15.7 g of Trizma HCl (Sigma-Aldrich, cat. no. T3253) in 75 mL distilled water. The final pH of the solution should be 7.7. Adjust to a final volume of 100 mL with distilled water.

COMMENTARY

Background Information

DNA is the genetic material, with two copies in each diploid cell. Accordingly, proper maintenance and propagation of DNA is necessary for the survival and existence of the organism. However, the DNA in a cell is being constantly assaulted by both exogenous and endogenous agents that may cause DNA damage (Searle, 1984). Although cells have specialized protection and repair systems to guard and maintain the integrity of the DNA, damage does occur. Some of the DNA damage may persist by either escaping the repair machinery or may simply be nonrepairable. This persistent damage may cause replication blockage, leading to cell death, or may be misread by the DNA polymerases and lead to mutations and cancer, as well as other disease states (Friedberg et al., 1995).

To elucidate the biological consequences of a DNA lesion, it is important to characterize its potential to induce mutations. Mutational analysis is generally studied in vitro by performing misincorporation studies (Guengerich, 2006). Misincorporation studies with DNA lesions are traditionally performed by DNA polymerase-catalyzed primer extension assays (Boosalis et al., 1987). In these assays a 5′-³²P end-labeled DNA primer is typically extended by a DNA polymerase along a DNA template containing the lesion of interest, either in the presence of all the four dNTPs or a single dNTP. The extended products are analyzed by polyacrylamide gel electrophoresis (PAGE; Ellington and Pollard, 1998). Kinetic analyses on single dNTP incorporation assays provide information regarding the preference of dNTP incorporation, while the full-length extension assays typically provides information about the ability of the DNA polymerase to read past and extend beyond the

lesion. PAGE provides information about the length of the extension products but not much about the fidelity of the DNA polymerization events (Boosalis et al., 1987). In fact, these approaches cannot distinguish between frameshift and blunt-end additions or incomplete primer extension events (Zang et al., 2005).

In order to gain better insight into the misincorporation events during translesion synthesis an alternative method, reported in this unit, was developed in our laboratory (Fig. 7.16.1; Zang et al., 2005). This method utilizes the use of LC-MS and LC-MS/MS for the analysis of the polymerase-catalyzed extension products, instead of PAGE. Similar to the full-length assay mentioned above, this method involves the extension of a primer containing one or more 2'-deoxyuridine (dU) residues by a DNA polymerase in the presence of all four dNTPs. The use of dU in the primer is a key feature of this method, which allows the extended primer to be cleaved at the site of the uracil using UDG, followed by cleavage of the primer into short pieces of DNA by hot piperidine, small enough for analysis by mass spectrometry. The short pieces of DNA that result from primer extension can be analyzed using LC-MS and LC-MS/MS to determine their nucleotide sequences and relative yields. This approach provides information regarding both the ability of the polymerase to read past and extend beyond the lesion and the fidelity of DNA synthesis—not only across the lesion but, also past the lesion in the subsequent polymerization steps (Zang et al., 2005; Christov et al., 2009).

LC-MS and LC-MS/MS Analysis of DNA

Small fragments of DNA can be easily detected and analyzed using LC-MS in the negative-ion ESI mode. Generally, small DNA fragments are observed as multiple charged species that can be deconvoluted to find $[M-H]^+$ ions. CID of DNA fragments results in the formation of various types of ions including the w and a-B ions (Ni et al., 1996). The w and a-B ions are formed from the cleavage of the glycosidic and the C3'-phosphate bonds (Fig. 7.16.2). These ions are not only the major ions observed but are consistently formed from fragmentation at every base of the sequence, generating a CID spectra that is characteristic of that particular sequence of DNA. Consequently, the w and a-B ions can be successfully used for sequencing of DNA fragments (Ni et al., 1996). The w and a-B ions can also be used for the detection of DNA modifications (Chowdhury and Guengerich, 2008, 2009).

Critical Parameters and Troubleshooting

The success of this experiment primarily depends on the yield of the various extension products and the limit of detection of the instrument used. Generally, translesion DNA synthesis is slow and often very inefficient, resulting in poor yields of the products. In addition, formation of multiple products further reduces the yield of each product. Although our general experience is that ~1 pmol of injected product is sufficient to obtain a good CID spectrum for sequence analysis in an LTQ instrument, the amount typically depends on the instrumentation and the length of the DNA fragment (Choi et al., 2006). Generally, the problem of limited yield is overcome by maximizing the yield of the extension products, using higher concentrations of primer template complex and DNA polymerases, and longer reaction times. Alternatively, use of mass spectrometers with increased sensitivity and resolution can be another solution.

Anticipated Results

Generally, the anticipated results are full-length extension products that are either error-free or contain varying amounts of one or more misincorporations (Figs. 7.16.3 and 7.16.4). Misincorporations may result from insertion of a noncomplementary base either opposite the lesion or across the neighboring bases. Occasionally -1, -2, +1, or +2 frameshift products are also observed (Fig. 7.16.4). The negative frameshift products are formed as a result of

skipping of the lesion and/or its neighboring base by the polymerase while the positive frameshift products results from addition of one or more bases across the lesion. Another type of product often observed is blunt-end additions, where an extra base is inserted at the end (these are not particularly relevant to the mechanisms of misincorporation; Fig. 7.16.4).

Time Considerations

The primer extension assays and the cleavage of the extension products can be accomplished in 2 days. LC-MS of the extension products may take from a few hours to 1 to 2 days, depending on the complexity of the extension products. The analysis of the MS data will take ~1 day. The authors suggest that a list of possible extension products and their corresponding multiply charged species should be created before performing the LC-MS analysis. This process will allow quick detection of DNA extension products for LC-MS/MS. The authors also suggest use of 2 to 3 scan events for LC-MS/MS data acquisition in case of the abundant ions.

Acknowledgments

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Figure 7.16.1.

Schematic representation of the approach used for the liquid chromatography-mass spectrometry analysis of DNA polymerase reaction products. The first step involves the extension of a primer containing one or more dU residues across a lesion containing template by a DNA polymerase in the presence of all four dNTPs. Following primer extension, the DNA is cleaved into small pieces in a two-step process to facilitate LC-MS analysis. dU in the primer is first removed using UDG. The abasic sites formed are then converted into cleavage sites by hot piperidine. The short pieces of DNA that resulted from primer extension are finally analyzed using LC-MS and LC-MS/MS to determine their nucleotide sequences and relative yields.

Figure 7.16.2.

CID of DNA fragments resulting in the generation of w and a-B ions (Ni et al., 1996). **(A)** CID fragmentation of specific bonds in DNA resulting in a-B and w ions. **(B)** Figure showing that fragmentation generally can occur at any base of the DNA fragment forming w_n and a_n-B_n ions that can be used to determine the sequence of the DNA fragment.

Figure 7.16.3.

Analysis of the extension product across O^6 -benzyl (Bz) G DNA lesion by human DNA polymerase η (Choi, 2006). (A) Total ion chromatogram (TIC) of the reaction mixture. (B) ESI-LC-mass spectrum of the products eluting at 3.90–4.2 min in TIC spectrum shown in Figure 7.16.3A. (C) TIC of the ESI-LC-MS/MS analysis of the m/z 1099.6 species. (D) CID mass spectrum of m/z 1099.6 species. (E) TIC of the ESI-LC-MS/MS analysis of the m/z 1103.3 species. (F) CID mass spectrum of the m/z 1103.3 species.

O⁶-BzG			
5 ' GCCUCGAGUCAGCCGUAGACGUAG			
3 ' CGGAGCTCGGTCGGCGTCTGCGTCG*CTCCTGCGGCT			
	AGGACGCCGA	1	%
	GAGGACGCCGA	2	%
C	GAGGACGCCGA	37	%
C	GAGGACGCCGAA	13	%
C	GAGGACGCCGAG	5	%
T	GAGGACGCCGA	23	%
T	GAGGACGCCGAA	8	%
A	GAGGACGCCGA	11	%

Figure 7.16.4.

Products of primer extension across O⁶-BzG DNA template by human DNA polymerase η and their relative yields (Choi et al., 2006).

Table 7.16.1

Gradient Program for Liquid Chromatography

Time (min)	% A	% B	Flow rate (μL/min)
0	100	0	150
3	97	3	150
4.5	80	20	150
5	0	100	150
5.5	0	100	150
6.5	100	0	150
9	100	0	150

Table 7.16.2

CID Fragments for 5'-pAGCGAGGACGCCGA-3' (from *O*⁶-BzG) (Choi et al., 2006)^a

N	Charge	a-B	w	y	d-H ₂ O
1	-1	330.059	250.093	312.049	
2	-1	490.051	659.111	579.145	641.101
	-2	244.521	329.051	289.068	320.046
3	-1	819.103	948.157	868.191	930.147
	-2	409.048	473.574	433.591	464.569
	-3	272.362	315.380	288.725	309.377
4	-1	1108.149	1237.203	1157.237	1259.199
	-2	553.571	618.097	578.114	629.096
	-3	368.711	411.729	385.074	419.061
5	-1	1437.202	1566.255	1486.289	1572.257
	-2	718.097	782.624	742.641	785.624
	-3	478.395	521.413	494.758	523.413
6	-1	1750.259	1855.301	1775.335	1901.309
	-2	874.625	927.147	887.164	950.150
	-3	582.747	617.762	591.106	633.097
7	-1	2079.311	2168.359	2088.393	2230.361
	-2	1039.151	1083.675	1043.692	1114.676
	-3	692.431	722.114	695.459	742.781
8	-1	2408.363	2497.411	2417.445	2543.418
	-2	1203.677	1248.201	1208.218	1271.205
	-3	802.116	831.798	805.143	847.134
9	-1	2721.421	2826.463	2746.497	2832.464
	-2	1360.206	1412.727	1372.744	1415.728
	-3	906.468	941.482	914.827	943.483
10	-1	3010.467	3139.520	3059.554	3161.517
	-2	1504.729	1569.256	1529.273	1580.254
	-3	1002.817	1045.835	1019.179	1053.167
11	-1	3359.519	3468.573	3388.607	3450.563

N	Charge	a-B	w	y	d-H ₂ O
12	-2	1669.255	1733.782	1693.799	1724.777
	-3	1112.501	1155.519	1128.863	1149.515
	-1	3628.565	3757.619	3677.653	3739.609
13	-2	1813.778	1878.305	1838.322	1869.300
	-3	1208.849	1251.867	1225.212	1245.864
	-1	3917.611	4086.671	4006.705	4068.661
	-2	1958.301	2042.831	2002.848	2033.826
	-3	1305.198	1361.551	1334.896	1355.548

^dThe numbers in bold represents the a-B and w ions that were detected in the CID spectra shown in Figure 7.16.3.

Table 7.16.3

CID Fragments for 5'-pAGTGAGGAGGCCGA-3' (from *O*⁶-Benzyl G) (Choi et al., 2006)^a

n	Charge	a-B	w	y	d-H ₂ O
1	-1		330.059	250.093	312.049
2	-1	490.051	659.111	579.145	641.101
	-2	244.521	329.051	289.068	320.046
3	-1	819.103	948.157	868.191	945.147
	-2	409.048	473.574	433.591	472.069
	-3	272.362	315.380	288.725	314.377
4	-1	1123.149	1237.203	1157.237	1274.199
	-2	561.070	618.097	578.114	636.595
	-3	373.711	411.729	385.074	424.061
5	-1	1452.201	1566.255	1486.289	1587.256
	-2	725.596	782.624	742.641	793.124
	-3	483.395	521.413	494.758	528.413
6	-1	1765.259	1855.301	1775.335	1916.309
	-2	882.125	927.147	887.164	957.650
	-3	587.747	617.762	591.106	638.097
7	-1	2094.311	2168.359	2088.393	2245.361
	-2	1046.651	1083.675	1043.692	1122.176
	-3	697.431	722.114	695.459	747.781
8	-1	2423.363	2497.411	2417.445	2558.418
	-2	1211.177	1248.201	1208.218	1278.705
	-3	807.115	831.798	805.143	852.134
9	-1	2736.420	2826.463	2746.497	2847.464
	-2	1367.706	1412.727	1372.744	1423.228
	-3	911.468	941.482	914.827	948.482
10	-1	3025.466	3139.520	3059.554	3176.516
	-2	1512.229	1569.256	1529.273	1587.754
	-3	1007.817	1045.835	1019.179	1058.167
11	-1	3354.518	3468.573	3388.607	3465.562

n	Charge	a-B	w	y	d-H ₂ O
12	-2	1676.755	1733.782	1693.799	1732.277
	-3	1117.501	1155.519	1128.863	1154.515
	-1	3643.564	3772.618	3692.652	3754.608
	-2	1821.278	1885.805	1845.822	1876.800
13	-3	1213.849	1256.867	1230.212	1250.864
	-1	3932.610	4101.670	4021.704	4083.660
	-2	1965.801	2050.331	2010.348	2041.326
	-3	1310.198	1366.551	1339.896	1360.548

^dThe numbers in bold represents the a-B and w ions that were detected in the CID spectra shown in Figure 7.16.3.