Chemiluminescent DNA Abasic (AP) Site Assay

APPLICATION

DNA abasic sites are a type of DNA damage generated by reactive oxygen species (oxidative damage). This method allows quantification of the number of DNA abasic sites per 10^5 base pairs of genomic DNA purified from coral tissue samples.

EQUIPMENT AND SUPPLIES

- DNA purification kit using NaI or guanidine/detergent lysis method (this example uses the GetPure® DNA kit, Dojindo Molecular Technologies)
- AP Site counting kit (example uses Dojindo DNA Damage Quantification kit)
- 1.5 ml sterile tubes
- Micropipettors (range 0.1-1000 µL)
- Multichannel pipettor (50-300 µL)
- Polyvinylpolypyrrolidone (PVPP)
- Heat block (65°C)
- Vortex mixer
- Liquid nitrogen or dry ice
- Gloves and face shield for use with liquid nitrogen
- Stainless steel spatulas
- Eppendorf tube rack
- Refrigerated tabletop centrifuge (14,000 rcf)
- Ethanol, molecular biology grade
- Vacuum concentrator or 37°C incubator
- Fluorometer for DNA quantification (example uses Invitrogen's Qubit® fluorometer)
- Luminometer (microplate capability, Biotek Synergy HT® used in this example)
- White, 96-well microplates (example uses Nunc Maxisorp ®)
- Tris-EDTA (TE) solution (10 mM Tris-1 mM EDTA, pH 8.0)
- Fluorometric DNA quantification kit (this example uses the Quant-iT HS® kit, Molecular Probes/Invitrogen)
- Refrigerated (4°C) storage
- Paper towels
- Parafilm®
- Chemiluminescent reagent (PerkinElmer Western Lightning Plus®, or similar)

PROCEDURE (24 coral samples will fill one 96-well plate)

I. Purification of DNA from coral tissue homogenate (**LINK**) (Dojindo GetPure® DNA Kit)

1. Using a small stainless steel spatula chilled in liquid nitrogen or dry ice, place 50 mg frozen coral tissue homogenate in sterile 1.5 mL tube containing 400 µL room temperature lysis buffer and ~15 mg polyvinylpolypyrrolidone (PVPP, Sigma).

2. Vortex to mix, then add 10 µL proteinase K. Vortex to mix and incubate at 65°C, 10 min.
3. Remove tube from water bath and let sit at RT for 2 min.
4. Add 2 µL RNase, vortex 5 s, and let sit at RT for 2 min.
5. Add 80 µL Precipitation solution I and vortex 5 s.
6. Add 80 µL Precipitation solution II and vortex 5 s.
7. Centrifuge sample at 14,000 rcf and 4°C for 10 min.
8. Remove supernatant to clean tube and add an equal volume (~400 µL) of cold 100% ethanol. Invert tube to mix then vortex for 5 s.
9. Centrifuge sample at 14,000 rcf and 4°C for 10 min.
10. Decant supernatant and add 1.0 mL 70% ethanol. Vortex to mix and spin in for 5 min at 14,000 rcf and 4°C.
11. Decant supernatant and wick dry on paper towel. Dry DNA in vacuum concentrator, low heat for 5 min, or in a 37°C incubator until liquid has evaporated (~20 min).
12. Resuspend DNA in 50-100 µL TE and store at 4°C for at least 4 h (overnight). This helps to make sure DNA is in solution and reduces errors in quantification.

II. Quantification of DNA (Molecular Probes Quant-iT HS® DNA kit, Invitrogen):

1. To 198 µL diluted working reagent, add 2 µL well-mixed DNA sample.
2. Dilute standards 1:20 in a 200 µL total volume of working solution.
3. Analyze standards first, then samples on the Qubit® reader, as instructed by the manufacturer.
4. Multiply concentration by 100 to get concentration of the original solution.

Note: Recovery of DNA should be at least 10 µg/ml.

III. Labeling DNA (Aldehyde Reactive Probe):

1. Dilute DNA samples of interest to a 10 µg/mL concentration. (One-tenth less DNA as compared to manufacturer's colorimetric protocol.)
2. Remove 10 µL DNA to a clean 0.5 mL tube and add 5 µL ARP solution from the Dojindo DNA Damage Quantification kit. Incubate in a 37°C water bath for 1 h.
3. Wash the inside of the filtration tube cup with 200 µL TE and spin quickly at 8000 rcf. Discard liquid in bottom of tube.
4. Add 385 µL TE to the ARP-DNA sample and transfer to the filtration tube.
5. Centrifuge at 2500 g for 15 min at room temperature. Discard filtrate solution.
6. Add 400 µL TE to the filter cup and resuspend DNA on the filter by pipetting up and down.
7. Centrifuge as in #5 above.

8. Add 200 µL TE to tube and resuspend DNA on the filter again. Transfer nucleic acid to a clean tube.
9. Add 200 µL more TE to the filter cup and wash well. Combine this with the DNA from step 8 (400 µL total volume).
10. Store ARP-labeled DNA at 4°C.

Note: While the kit instructions state that the labeled DNA is stable for up to 6 months, it is best to use as soon as possible for the most consistent results.

IV. Binding DNA to Microtiter Plate

1. Dilute 90 µL of ARP-labeled DNA with 310 µL TE.
2. Dilute AP standards 5 and 10 (5 and 10 AP sites/10^5 bp), 1:10 with TE for lower end of standard curve
3. Plate 60 µL of both standards and up to 24 samples (S1-S24) in triplicate in wells of white microtiter plate, using TE for the blanks (See Fig. 1). Samples should be in random order, especially if evaluating samples from several sites. Note: standards will be 0.5 (dil), 1.0 (dil), 2.5, 5.0, 10.0, 20.0, and 40.0 AP sites/10^5 bp. Do not use the zero standard.
4. Add 100 µL DNA binding solution to each well with a multichannel pipettor; cover with parafilm and let sit at RT overnight.

FIGURE 1. Plate layout for DNA AP site assay. Standards are in columns (red) and samples designated with an "S" are plated horizontally in triplicate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	**TE**	S1	S1	S1	**1**	S9	S9	S9	**10**	S17	S17	S17
B	**TE**	S2	S2	S2	**2.5**	S10	S10	S10	**10**	S18	S18	S18
C	**TE**	S3	S3	S3	**2.5**	S11	S11	S11	**20**	S19	S19	S19
D	**0.5**	S4	S4	S4	**2.5**	S12	S12	S12	**20**	S20	S20	S20
E	**0.5**	S5	S5	S5	**5**	S13	S13	S13	**20**	S21	S21	S21
F	**0.5**	S6	S6	S6	**5**	S14	S14	S14	**40**	S22	S22	S22
G	**1**	S7	S7	S7	**5**	S15	S15	S15	**40**	S23	S23	S23
H	**1**	S8	S8	S8	**10**	S16	S16	S16	**40**	S24	S24	S24

V. DNA AP Site Assay

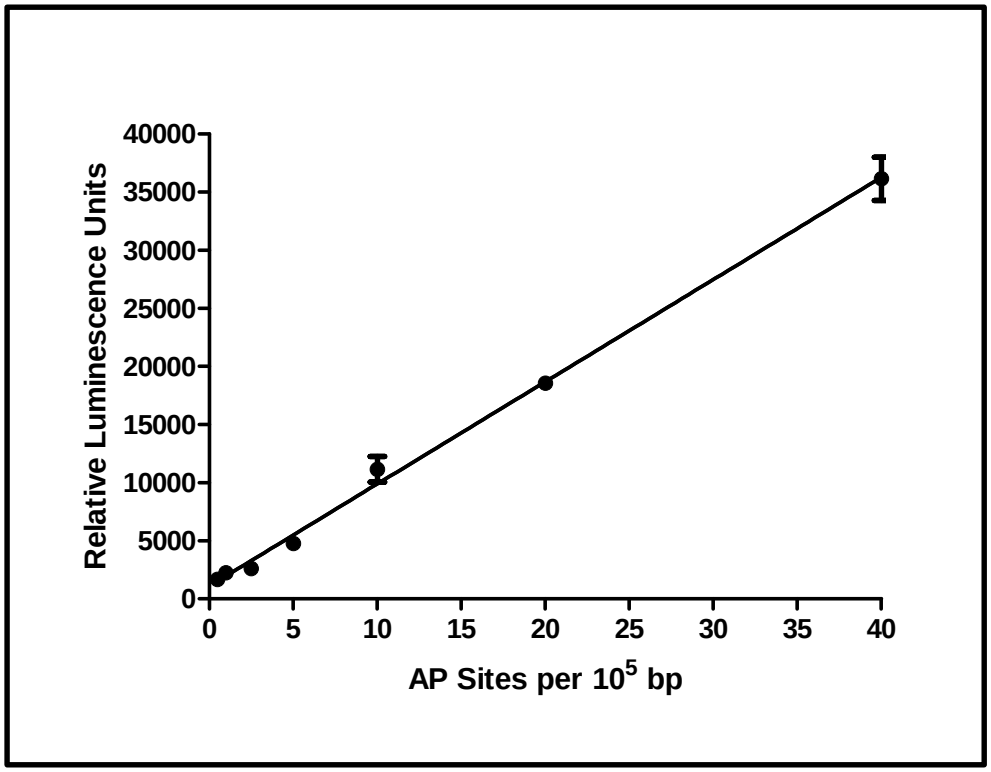
1. Remove luminol and oxidizer (e.g., Western Lightning® Chemiluminescent reagent, PerkinElmer) from refrigerator and let come to room temperature.
2. Remove binding solution from each well and wash with 250 µL Dojindo washing buffer 5 times. A plate washer may be substituted for manual washing, but will need to be optimized for the assay.
3. Tap plate upside-down on paper towels to dry wells.
4. Make up HRP-streptavidin solution (3.75 µL in 15 mL washing buffer will do one plate) and add 150 µL to each well.
5. Cover plate with parafilm and incubate at 37°C for 1 h.
6. Remove liquid from wells and wash 5X as in steps 2-3 above.
7. Set up plate reader and read blank plate. NOTE: The plate should be from the same lot as the plate with samples.
8. Tap sample plate on paper towels to dry.
9. Make up 12 mL of a 1:1 solution of luminol:oxidizer from the Western Lightning kit and add 100 µL to each well.
10. Read plate immediately Lum/E, sensitivity 150 and 180 on luminometer.
11. Calculate actual DNA AP sites by multiplying all results by a factor of 10, since one tenth the amount of DNA was used as compared to the manufacturer's protocol.

EXPECTED RESULTS

I. Standard curve

The linear regression analysis (**LINK**) results in a standard curve (calculated for you on typical software associated with the luminometer) which should look similar to Fig. 2. It is often easier to have the luminometer calculate the 10-fold dilution for you. This is easily done by multiplying each standard by a factor of 10 and recording that information on the instrument. The standard curve will then run from 5 to 400 AP sites (instead of 0.5 to 40).

FIGURE 2. DNA AP site standard curve. Results from a typical experiment with a luminometer sensitivity setting of 150. The r^2 value=0.99.



Note: The r^2 value should be at least 0.95, with a routine value of >0.98.

II. Coral Data

The spreadsheets from a typical coral sample assay (*Acropora palmata*) evaluated with a luminometer sensitivity setting of 150 are shown in Figs. 3-5. Note that background (TE blank in red) averages 279 in Fig. 3. This background should be subtracted from all sample values prior to calculating AP site numbers from the standard curve (Fig. 4). From the standard curve, AP site values can be calculated for each coral sample. Data analysis software is associated with many luminometers, so this may be automatically done for you. A typical coral sample AP site evaluation for the above data sets is in Fig. 5. The 10-fold dilution has been recorded on the instrument as detailed above, so no further calculations are required. Samples with values above the limit of the standard curve (e.g., numbers 3, 8, 10, and 14, concentrations >419.75) or with high %CV should be re-analyzed. These samples can be diluted and the assay repeated as detailed in the AP site kit instructions, or researchers may request that the kit manufacturer synthesize wide range standards for samples with relatively high numbers of DNA abasic sites.

FIGURE 3. Initial luminometer values for the DNA AP site assay. Background (TE blank) values are indicated in red and remaining AP standards are shown in blue. Raw values for individual corals are in black.

	1	2	3	4	5	6	7	8	9	10	11	12
A	**338**	23979	23867	28698	**577**	2285	2469	2617	**5725**	4439	3920	3758
B	**265**	3023	3704	3334	**856**	27436	27124	25986	**5883**	1919	1884	2308
C	**236**	66175	72366	73573	**644**	4198	4505	4947	**11580**	7081	6906	7445
D	**243**	1782	1750	1818	**622**	1828	2043	2125	**11421**	4587	4202	4158
E	**211**	764	769	863	**1394**	6779	6991	8533	**13315**	9300	9496	10025
F	**190**	11567	12570	13768	**1401**	50404	49853	54988	**22813**	2210	2381	2418
G	**384**	487	639	664	**1369**	2693	3020	3174	**28592**	2990	3007	2960
H	**380**	29204	27470	31739	**3840**	1681	1819	2000	**24892**	11783	12415	10766

FIGURE 4. Luminometer readings for AP site assay with background correction incorporated.

	1	2	3	4	5	6	7	8	9	10	11	12
A	**58**	23700	23588	28417	**299**	2005	2191	2337	**5445**	4156	3642	3477
B	**-13**	2743	3424	3056	**577**	27155	26843	25704	**5604**	1640	1606	2030
C	**-45**	65896	72088	73295	**367**	3920	4226	4667	**11301**	6804	6626	7163
D	**-36**	1503	1471	1540	**341**	1549	1765	1847	**11141**	4306	3924	3879
E	**-67**	486	491	585	**1115**	6500	6713	8254	**13037**	9022	9218	9746
F	**-89**	11289	12292	13490	**1122**	50125	49574	54710	**22536**	1929	2103	2139
G	**105**	208	362	385	**1090**	2414	2742	2896	**28314**	2712	2730	2681
H	**102**	28924	27193	31460	**3562**	1402	1542	1722	**24612**	11504	12136	10484

FIGURE 5. Typical coral sample AP Site assay results. Means are reported for the triplicate samples.

Well ID	Conc.\Dil.	Well	M#1 Lum/E Corr.	Concentrations	Nb	Mean	Std Dev	CV (%)
SPL1		A4	28417	>419.750	2	380.79	1.217	0.32
		A2	23700	381.65				
		A3	23588	379.93				
SPL2		B2	2743	59.515	3	64.608	5.24	8.11
		B3	3424	69.983				
		B4	3056	64.326				
SPL3		C2	65896	>419.750				
		C3	72088	>419.750				
		C4	73295	>419.750				
SPL4		D2	1503	40.454	3	40.48	0.531	1.311
		D3	1471	39.962				
		D4	1540	41.023				
SPL5		E2	486	24.821	3	25.354	0.857	3.381
		E3	491	24.898				
		E4	585	26.343				
SPL6		F2	11289	190.88	3	207.3	16.938	8.171
		F3	12292	206.3				
		F4	13490	224.71				
SPL7		G2	208	20.548	3	22.244	1.479	6.651
		G3	362	22.915				
		G4	385	23.269				
SPL8		H2	28924	>419.750				
		H3	27193	>419.750				
		H4	31460	>419.750				
SPL9		A6	2005	48.171	3	50.825	2.558	5.033
		A7	2191	51.03				
		A8	2337	53.274				
SPL10		B6	27155	>419.750	1	412.46		
		B7	26843	>419.750				
		B8	25704	412.46				
SPL11		C6	3920	77.607	3	83.002	5.772	6.954
		C7	4226	82.31				
		C8	4667	89.089				
SPL12		D6	1549	41.161	3	43.795	2.366	5.403
		D7	1765	44.481				
		D8	1847	45.742				

Well ID	Conc.\Dil.	Well	M#1 Lum/E Corr.	Concentrations	Nb	Mean	Std Dev	CV (%)
SPL13		E6	6500	117.27	3	127.34	14.712	11.553
		E7	6713	120.54				
		E8	8254	144.23				
SPL14		F6	50125	>419.750				
		F7	49574	>419.750				
		F8	54710	>419.750				
SPL15		G6	2414	54.458	3	58.608	3.784	6.457
		G7	2742	59.499				
		G8	2896	61.867				
SPL16		H6	1402	38.902	3	41.259	2.466	5.977
		H7	1542	41.054				
		H8	1722	43.82				
SPL17		A10	4156	81.234	3	75.122	5.444	7.246
		A11	3642	73.334				
		A12	3477	70.797				
SPL18		B10	1640	42.56	3	44.384	3.621	8.159
		B11	1606	42.037				
		B12	2030	48.555				
SPL19		C10	6804	121.94	3	122.87	4.205	3.422
		C11	6626	119.2				
		C12	7163	127.46				
SPL20		D10	4306	83.54	3	79.395	3.606	4.542
		D11	3924	77.668				
		D12	3879	76.977				
SPL21		E10	9022	156.03	3	160.75	5.756	3.581
		E11	9218	159.05				
		E12	9746	167.16				
SPL22		F10	1929	47.002	3	48.97	1.726	3.525
		F11	2103	49.677				
		F12	2139	50.23				
SPL23		G10	2712	59.038	3	58.972	0.381	0.646
		G11	2730	59.315				
		G12	2681	58.562				
SPL24		H10	11504	194.18	3	192.2	12.813	6.667
		H11	12136	203.9				
		H12	10484	178.51				

Note: The percent CV values should be less than 10%. Often these values are lower than 5%.

Table 1. AP site data collected from a variety of wild and captive coral. The number of AP sites are reported as the mean from several coral colonies (n=2-8). (CCCRF=Coral Culture and Collaborative Research Facility, NOS/NOAA/CCEHBR, Charleston, SC)

Species (n)	AP Sites (mean per 10^5 bp) ± SEM	Collection Site
Porites astreoides (5)	129 ± 19	St. John, USVI
Porites divaricata (3)	364 ± 98	CCCRF
Porites lobata (7)	172 ± 35	Maui, HI
Acropora chesterfieldensis (3)	98 ± 38	CCCRF
Acropora millepora (2)	67± 16	CCCRF
Acropora palmata (8)	80 ± 12	Guantanamo, Cuba
Montastraea annularis (3)	387 ± 70	CCCRF
Leptogorgia sp. (3)	34 ± 9.0	Gray's Reef, SC
Nematostella vectensis (3)	30 ± 4.0	CCCRF
Aiptasia sp. (3)	229 ± 65	CCCRF

LINKS

www.dojindo.com
www.perkinelmer.com
www.biotek.com
www.invitrogen.com
www.nuncbrand.com

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Submitted by: Lisa A. May
NOS/NOAA/ CCEHBR
Coral Health and Disease Program
Charleston, SC
Last updated: 10-7-2011
Contact: CDHC.Coral@noaa.gov