3050 Spruce Street
Saint Louis, Missouri 63103 USA
Telephone (800) 325-5832 (314) 771-5765
Fax (314) 286-7828
email: techserv@sial.com
sigma-aldrich.com

ProductInformation

ENDONUCLEASE VIII AND III SUBSTRATE SET

Product Number **E 7651**
Storage Temperature –20 °C

Product Description

This set contains a 33 base 5,6-DHT mutated oligonucleotide and a 33 base normal complementary oligonucleotide necessary to produce a radiolabeled ds-oligonucleotide substrate for Endonuclease VIII & III.

Specifically mutated double stranded (ds) oligonucleotide are replacing irradiated or chemically oxidized DNA as substrates for DNA repair proteins. The reason for this switch is the simplicity of mutated ss-oligonucleotide preparation by automated oligonucleotide synthesizers, the specificity of the assay and the simplicity of the detection methods.[1]

The procedure requires radioactive labeling of the oligonucleotide substrate. The mutated 5,6-dihydrothymine (DHT) oligonucleotide is 5'- ^{32}P labeled and then annealed to a normal complementary oligonucleotide. The DNA repair enzymes are able to cleave the ds-oligonucleotide substrate at the mutated nucleotide, which is located at the middle of the labeled strand.[2] The fragments obtained are denatured and separated on an acrylamide-urea gel, which is then exposed to X-ray film.

Typically one preparation of the ^{32}P radiolabeled substrate is made with 100 pmol of mutated oligonucleotide strands and 130 pmol of complementary oligonucleotide strands. This preparation is sufficient for approximately 100 enzymatic tests. The substrate radioactivity decays with time ($t_{1/2}$= 14 days). ^{32}P-radiolabeled substrate may be used for 4 weeks at the most.

The Endonuclease VIII & III Substrate Set contains oligonucleotides for 3 preparations of radiolabeled ds-oligonucleotide (100 enzymatic tests each).

Components

E 9401	Endonuclease VIII & III Substrate-5,6-Dihydrothymine Strand (Endo VIII DHT Strand), vacuum dried, 350 pmol. **1 vial**. 33 bases, 5,6 DHT at position 16
E 2026	Endonuclease VIII & III Substrate, Complementary Strand (Endo VIII Substrate Comp. Strand), vacuum dried 450 pmol. **1 vial**. 33 bases

Storage/Stability

Store dessicated at –20 °C.

Reconstitution Instructions

1. Reconstitute Endo VIII DHT Strand (Product Code E 9401) with 35 µl molecular biology grade deionized water.
   Store at –20 °C.
2. Reconstitute Endo VIII Substrate Comp. Strand (Product Code E 2026) with 45 µl molecular biology grade deionized water. Store at –20 °C.

<u>Equipment and Reagents Needed but not Provided</u>

- Endo VIII Product Code E0651 or other DNA repair enzyme.
- T4 polynucleotide kinase (PNK) (Product Code P4390).
- T4 polynucleotide kinase (PNK) buffer.
- γ ^{32}P-ATP 10mCi/ml
- Endo VIII dilution buffer:
  20 mM Tris, pH 7.5, 10% glycerol, 100 mM NaCl, 0.5 mM EDTA, 1 mM DTT.
- 10X Reaction buffer:
  0.5 M Tris, pH 7.5, 0.5 M KCl, 20 mM EDTA.
- Stop solution:
  90 % formamide, 0.1 % w/v bromophenol blue, 0.1 % xylene cyanole, 20 mM EDTA, pH 8.
- G-25 microspin column.
- 20% denaturing (7 M urea) acrylamide gel and electrophoresis apparatus.[3]
- Running buffer:
  TBE: 89 mM TRIS, 2 mM EDTA, 89 mM Boric acid, pH 8.
- X-ray film and developing machine.

Procedure

Principle of Assay

The assay is based on the ability of DNA repair proteins such as Endonuclease VIII to cleave 5,6 dihydrothymine (DHT) mutated double strand oligonucleotide. The DHT strand is first labeled with 5' ^{32}P and then annealed to its complementary strand to form the Endonuclease VIII substrate.

Endonuclease VIII & III recognize and remove the mutated base (DHT), then cleave the DNA at its Apyrimidinic (AP) site (lyase activity). Denaturation of the double strand and separation on denatured polyacrylamide-urea gel (20%, 7M urea) produce two labeled bands; a 15/mer band,[4] which is the cleavage product and a 33 oligonucleotide as residual uncleaved substrate.

Note: Use molecular biology grade water

Radiolabeled Substrate Preparation

Labeling of DHT Mutated Strand

1. Prepare the following mix

Reagent	
10X PNK buffer	3 µl
5,6 DHT strand oligonucleotide	10 µl (100 pmol)
ATP γ-^{32}P 10 mCi/ml	3 µl (30 µCi)
T4 PNK	1 µl
Deionized H_2O	13 µl

2. Incubate for 60 min at 37 °C.
3. Inactivate for 10 min at 70 °C.
4. Clean sample from remaining ATP on G-25 microspin column according to manufacturer instructions (about 30 µl elution).

Annealing to the Complementary Strand

1. Add 13 µl (130 pmol) of complementary strand (Product Code: E 2026).
2. Anneal strands by incubation: 2 min. at 75 °C and then slowly cool to room temperature (this procedure was carried out over 2 hrs in a water bath).
3. Store labeled substrate at –20 °C in a radioactive protected box.

Enzymatic Assay Procedure

1. Prepare 20% denaturing gel (7 M urea) and assemble the electrophoresis apparatus.
2. Dilute Endo VIII enzyme using the dilution buffer.
3. Prepare reaction mix for 10 reactions

Reagent	Amount per 10 reactions
10X reaction buffer	10 µl
Labeled Endo III substrate	2 µl
Deionized H_2O	68 µl

4. Dispense 8 µl of reaction mix to each tube.
5. Start the reaction by the addition of 2 µl diluted enzyme samples with 20 seconds intervals. For control add 2 µl of dilution buffer in place of enzyme.
6. Incubate for 10 min. at 30 °C.
7. Stop reactions by the addition 5 µl stop solution.
8. Boil for 5 min. at 95 °C.
9. Load 4 µl sample on the denaturing gel. Note: wash the wells before loading.
10. Run the mini gel at 200V with circulating cold water (~10ºC) to reduce heating until the stain front reaches 1-2 cm of the bottom of the gel (bromophenol blue and xylene cyanole run as 8 and 28 bases respectively on 20% denaturing gels).
11. Carefully disassemble the gel and lay it on a piece of Whatman 3 mm paper.
12. Cover the gel with a sheet of plastic wrap. Note: do not dry the gel, it may crack.
13. Expose to X-ray film for 16 hr. at –20 °C. It is recommended to put two layers of film on the gel in order to get at least one gel properly exposed.

References

1. Harrison, L., et al., Nucleic Acids Research, **26**, 932-941 (1998).
2. D'Ham, C., et al., Biochemistry, **38,** 3335-3344 (1999).
3. Current Protocols in Molecular Biology, Wiley, 2.12.

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