EURx® MOLECULAR BIOLOGY PRODUCTS

Endocyclase N15

Protelomerase N15 (TelN)
Escherichia coli phage N15

Cat. No.	size
E4410-01	50 units
E4410-02	250 units

Unit Definition:

One unit is the amount of enzyme required to process 90% of 1 pmol cleavable substrate DNA in a 20 µl reaction volume within one hour at 25°C.

Storage Conditions:

Store at -20°C.

The enzymatic activity is stable at least for 1 year.

Reaction Temperature:

25°C (recommended) – 30°C

Inactivation:

- DNA purification (EURx PCR / DNA Clean up Kit (E3520) or 3 in 1 Basic DNA Kit (E3545))
- Heat inactivation + SDS

The enzyme exhibits high affinity towards DNA. Thorough DNA purification is mandatory.

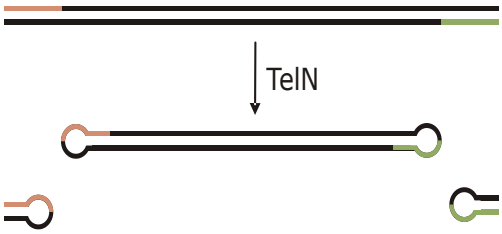

Fig. 1: TelN acting on linear DNA (e.g. PCR amplicons).

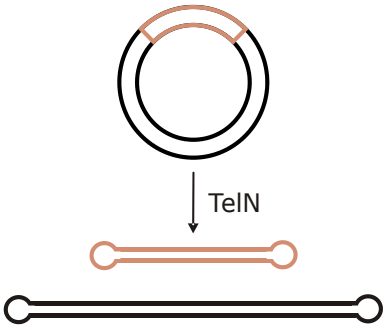

Fig.2: TelN acting on covalently closed DNA (e.g. plasmid DNA).

Endocyclase N15 catalyzes the formation of covalent links between complementary 5'-and 3'-ends and forms hairpin ends at a specific double-stranded DNA recognition site.

Description:

- Endocyclase N15 (protelomerase, "prokaryotic telomerase" TelN), a dedicated DNA cleaving-rejoining enzyme, forms site-specific covalent links between 3'- and 5'- ends of complementary DNA strands.
- The enzyme exhibits a topoisomerase IB and tryosine recombinase/ resolvase (Y recombinase) mode of action for DNA cleavage and rejoining. Whereas canonical recombinases act on two different DNA sites, endocyclase catalyzes hairpin formation between both opposite strands of a single dsDNA recognition site.
- In a process coined as "telomere resolution", the enzyme cleaves a 56 bp imperfect palindromic sequence (the telomere resolution site) and rejoins 5'- and 3'-ends under formation of a covalently closed hairpin structure (1). Shorter versions of the recognition sequence serve as substrates (min. length: 36 bp), albeit cleavage efficiency decreases with recognition sequence fragment length.
- Processing completes with approx. 90% efficiency (10% of substrate DNA remains non-processed). Non-processed DNA molecules are susceptible to attack by double-strand specific nucleases.
- Covalently linked (capped) DNA hairpins are resistant to exonuclease digestion, as opposed to nuclease-sensitive, open-ended DNA (3).
- Useful for either converting PCR products or plasmids to linear plasmids with capped ends (e.g. for generation of linear BACs by PCR (3)) or for covalently clamping both strands of a PCR product.
- TelN processed linear plasmids are functional in mammalian cells. Such linear plasmids are used to introduce genes with high stability and a minimum of – potentially problematic - foreign nucleotide sequences (2). Linear, exonuclease-resistant plasmids are thus generated by the sole use of *in-vitro* technologies, such as PCR. Cultivation steps in bacteria are not necessary. This is advantageous for certain pharmaceutical applications such as gene therapy, where introduction of any material originating from living cells into production processes needs to be avoided.

Quality Control:

All preparations are assayed for contaminating exonuclease, endonuclease, for nonspecific RNase and single- and double-stranded DNase activities.

References:

1. *Deneke J. et al. (2000) PNAS 97 (14): 7721–7726.*
2. *Heinrich J. et al. (2002) J mol Med 80: 648–654.*
3. *Ooi Y.-S. et al. (2008) Plasmid 59: 63–71.*
4. *Markham N. R., Zuker, M. (2008) In: Keith, J. M., editor, Bioinformatics, Volume II; 453 Meth Mol Biol, Ch 1, 3–31.*

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Endocyclase N15 – Telomere Resolution Protocol

Mix the following components:

DNA substrate	1 pmol
10 x Endocyclase Buffer	2 µl
Endocyclase	1 U
H_2O, DNA and DNase free	to 20 µl
Total Volume: 20 µl	

- Add Endocyclase as last component to the reaction mix.
- Incubate for 30 min at 25°C (recommended) or at 30°C.
- Purify DNA and analyze by Agarose gel electrophoresis.

Direct processing of PCR products without prior purification

Endocyclase shows robust performance in various molecular buffers and is compatible with EURx PCR buffer systems. For direct processing, PCR reactions are supplemented with appropriate volumes of 10 x Endocyclase Buffer followed by endocyclase addition. Best results are obtained when target DNA is purified prior to processing.

PCR primers are not substrates for Endocyclase

Plain PCR primers with 5'-endocyclase recognition sequence extensions do not serve as substrates for endocyclase, because the palindromic recognition sequence lacks a second strand. Therefore non-incorporated primers do not contribute to the (calculation of) total amount of processable substrate.

Note 1:

Endocyclase exhibits high affinity towards the cleaved and rejoined DNA recognition site, since its *in vivo* function is to serve as additional protection for generated hairpin ends. Reaction products require thorough purification to ensure that no enzyme remains bound to DNA. Heat denaturation is generally not sufficient for full removal of denatured enzyme from DNA. Addition of denaturing reagents such as SDS is mandatory, eventually followed by additional purification steps such as phenol-chloroform extraction. A convenient and time saving alternative is to purify DNA with the GeneMatrix PCR/ DNA Clean up kit (Cat.No. E3520) or 3 in 1 Basic DNA Kit (E3545), which separate DNA efficiently from endocyclase.

Note 2:

The reaction proceeds quickly (> 90% of cleavable substrate turnover after 5 – 10 min) and without a detectable lag phase under standard reaction conditions (1).

Note 3:

If reaction is not performed with the provided reaction buffer:

- Telomerase-resolvase activity requires the presence of Ca^{2+} or certain other bivalent cations such as Mn^{2+}, Co^{2+} , Mg^{2+}, and alkaline earth metal ions, preferably with large ionic radius in concentrations between 5 mM and 10 mM (1).
- The recommended ionic concentration is 20 mM. High concentrations of certain anions (chloride > 200 mM, phosphate > 50 mM) inhibit enzymatic activity completely (1).
- The pH optimum is 7.5, good performance is obtained in the range between pH 6–10 (1).

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Calculation of pmol from µg DNA (double stranded DNA, dsDNA)

$$DNA\,[pmol] = \frac{DNA\ amount\ [\mu g] \cdot 10^{6}\ [\mu g\ g^{-1}]}{DNA\ length\ [bp] \cdot 616\ [g\ mol^{-1}\ bp^{-1}] \cdot 10^{12}\ [pmol\ mol^{-1}]}$$

Calculation of µg from pmol dsDNA

$$dsDNA\ amount\ [\mu g] = \frac{dsDNA\ [pmol] \cdot 616\ [g\ mol^{-1}\ nt^{-1}] \cdot dsDNA\ length\ [nt] \cdot 10^{6}\ [\mu g\ g^{-1}]}{10^{12}\ [pmol\ mol^{-1}]}$$

1 pmol dsDNA =	100 bp	61.6 ng
	500 bp	308 ng
	1 000 bp	616 ng
	2 500 bp	1.54 µg
	10 000 bp	6.16 µg
	20 000 bp	12.32 µg

One endocyclase recognition site per molecule: 1 pmol DNA = 1 pmol DNA substrate,

two endocyclase recognition sites per molecule: 1 pmol DNA = 2 pmol DNA substrate

(0.5 pmol DNA = 1 pmol DNA substrate), etc ...

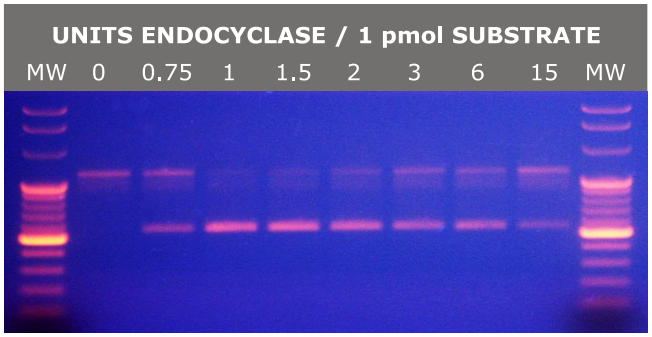

Fig.3: Processing of a 1 kb PCR product with a central recognition site into two 500 bp DNA fragments, which are covalently linked at one side. Endocylase N15 processing efficiency is highly dependent on the amount of enzyme. Best results are obtained, when exactly 1 U of enzyme is used per pmol of DNA.

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Endocyclase Processing of Genomic DNA / Plasmid DNA:

N15 endocyclase acts as dimer. It recognizes and generates a staggered cut at a required recognition sequence stretch, generating a proposed 6 bp overhang (1), Sequence stretches TelR and TelL are required for endocyclase substrate binding. Shortening of both stretches to meet specific experimental requirements (min. total length 36 bp) is possible at the cost of gradually reduced processing efficiency (1).

a) Before cleavage-rejoining of genomic DNA / plasmid DNA (small letters: non-palindromic region):

```
5'- TA TCA GCA CAC AAT tgc  CC ATT ATA | CGC GCG TAT AAT GG  act ATT GTG TGC TGA TA -3'
                                       ----------
3'- AT AGT CGT GTG TTA acg  GG TAA TAT GCG CGC | ATA TTA CC  tga TAA CAC ACG ACT AT -5'
    [          TelR          ] [  Recognition, Cleavage - Rejoining  ] [          TelL          ]
```

b) After cleavage-rejoining of genomic DNA / plasmid DNA:

```
                                    CG   CG
5'- TATCAGCACACAATtgcCCATTATA  C G  TATAATGGactATTGTGTGCTGATA -3'

3'- ATAGTCGTGTGTTAacgGGTAATATGCG CGCATATTACCtgaTAACACACGACTAT -5

    [          TelR          ]                    [          TelL          ]
```

PCR Primer Design for Inclusion of a Endocyclase Processing Site.

For insertion of a endocyclase cleavage site via PCR, the gene-of-interest specific primer sequence is extended with an imperfect palindromic sequence resembling the following structure:

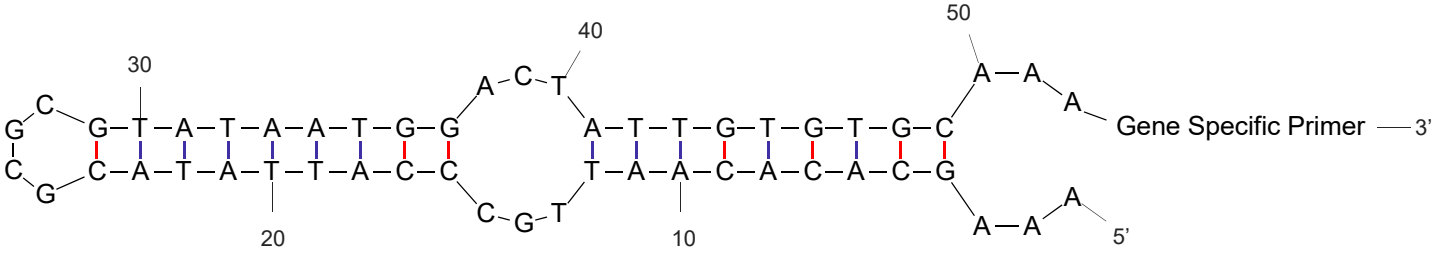
```
  [Ext]   [          > Palindrome >          ]   [          < Palindrome <          ]   [Ext]
5'- AAA   GCA CAC AAT TGC CC ATT ATA CGC    GCG TAT AAT GG ACT ATT GTG TGC    AAA gene specific primer -3'
    [     <Removed after Processing<     ]
```

This 46 bp recognition sequence is shorter than the native 56 bp palindromic sequence, but is recognized and cleaved with high efficiency. Cleavage and rejoining occurs between the endocyclase site included in the primer and the newly synthesized, primer-complementary DNA stretch. Following endocyclase processing, the inverted repeat is cleaved within the recognition sequence, leaving covalently linked, capped hairpins. Bases 1-23 at the 5'-primer terminus (including any 5'-label) are 3'-covalently linked to the 5'-end of the 29 3'-terminal nucleotides from the complementary strand and are removed after telomerase-resolvase processing as a covalently linked 52 nt / 26 bp DNA fragment.

Primer Design, General Considerations:

- **Non-Matching 5'- and 3'-Termini:** To prevent 5'-exonuclease activity of Taq DNA Polymerase, the 5'- and 3'-termini framing the endocyclase recognition sites must not match. In principle, the, 5'-AAA... and ...AAA-3' in the example sequence given above, are exchangeable with alternate sequences, as long as at least the three 5'- and 3'- terminal nucleotides do not match. For instance, it may be convenient to replace ...AAA-3' with a gene specific primer sequence to keep the overall primer sequence as small as possible. In this case, the 5' end must be adjusted accordingly.
- **Built-In Mismatches:** The suggested non-perfect palindromic primer extension contains a intentionally mismatched region. This mismatch is tolerated by endocyclase. Taq DNA polymerase has low strand-displacing activity and may have difficulties reading through extended hairpin stretches. Additionally, the mismatch prevents extensive formation of PCR artifacts such as primer dimers / multimers during PCR due to extended self complementary sequence stretches.
- **5'-Labelling:** Success of the reaction is monitored by adding a fluorescent label to the 5' end of the primer. After endocyclase cleavage and processing, the fluorescent label is released along with the 5'-terminal nucleotides and, upon agarose gel electrophoresis, migrates with the primer front. In practice, Fluorescein may be used as a convenient, PCR-stable label (excitation 494 nM [blue light or UV illumination], emission 521 nM [green light]).

Endocyclase PCR primer secondary structure (at room temperature under reaction conditions):



Calculation of secondary structure by use of mfold, Ref. 5

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