

## Claims

1. A terminal deoxynucleotidyl transferase (TdT) variant comprising an amino acid sequence at least ninety percent identical to an amino acid sequence selected from SEQ ID NO: 2, 5, 8, 11, 14, 17, 20, 23 26, 29, 32, 35, 38, 41, 44 or 47, wherein:
- 5 with respect to SEQ ID NO: 2 leucine at position 61 is substituted, cysteine at position 170 is substituted, arginine at position 204 is substituted, arginine at position 326 is substituted and glycine at position 329 is substituted;
- with respect to SEQ ID NO: 5 leucine at position 48 is substituted, cysteine at position 10 158 is substituted, arginine at position 192 is substituted, arginine at position 314 is substituted and glycine at position 317 is substituted;
- with respect to SEQ ID NO: 8 leucine at position 61 is substituted, tyrosine at position 171 is substituted, arginine at position 205 is substituted, arginine at position 327 is substituted and glutamic acid at position 329 is substituted;
- 15 with respect to SEQ ID NO: 11 leucine at position 61 is substituted, tyrosine at position 171 is substituted, arginine at position 205 is substituted, arginine at position 324 is substituted and glutamic acid at position 327 is substituted;
- with respect to SEQ ID NO: 14 glycine at position 61 is substituted, arginine at position 205 is substituted, arginine at position 327 is substituted and glutamic acid at position 20 330 is substituted;
- with respect to SEQ ID NO: 17 arginine at position 61 may or may not be substituted, alanine at position 158 is substituted, arginine at position 192 is substituted, arginine at position 311 is substituted and glutamic acid at position 314 is substituted;
- with respect to SEQ ID NO: 20 arginine at position 61 may or may not be substituted, 25 proline at position 171 may or may not be substituted, arginine at position 205 is substituted, threonine at position 321 is substituted and glutamic acid at position 324 is substituted;
- with respect to SEQ ID NO: 23 arginine at position 61 may or may not be substituted, alanine at position 171 may or may not be substituted, arginine at position 205 is substituted, alanine at position 322 may or may not be substituted and glutamic acid at position 325 is 30 substituted;

with respect to SEQ ID NO: 26 arginine at position 61 may or may not be substituted, alanine at position 171 may or may not be substituted, arginine at position 205 is substituted, arginine at position 322 is substituted and glutamic acid at position 325 is substituted;

5 with respect to SEQ ID NO: 29 methionine at position 48 is substituted, cysteine at position 158 is substituted, arginine at position 192 is substituted, arginine at position 310 is substituted and glutamic acid at position 313 is substituted;

with respect to SEQ ID NO: 32 methionine at position 61 is substituted, cysteine at position 171 is substituted, arginine at position 205 is substituted, arginine at position 323 is substituted and glutamic acid at position 326 is substituted;

10 with respect to SEQ ID NO: 35 methionine at position 61 is substituted, cysteine at position 171 is substituted, arginine at position 205 is substituted, arginine at position 323 is substituted and glutamic acid at position 326 is substituted;

with respect to SEQ ID NO: 38 methionine at position 61 is substituted, cysteine at position 171 is substituted, arginine at position 205 is substituted, arginine at position 323 is substituted and glutamic acid at position 326 is substituted;

15 with respect to SEQ ID NO: 41 methionine at position 61 is substituted, cysteine at position 171 is substituted, arginine at position 205 is substituted, arginine at position 323 is substituted and glutamic acid at position 326 is substituted;

20 with respect to SEQ ID NO: 44 methionine at position 48 is substituted, cysteine at position 158 is substituted, arginine at position 192 is substituted, arginine at position 309 is substituted and glutamic acid at position 312 is substituted; and

with respect to SEQ ID NO: 47 methionine at position 61 is substituted, cysteine at position 171 is substituted, arginine at position 205 is substituted, arginine at position 323 is substituted and glutamic acid at position 326 is substituted; and

25 wherein the TdT variant (i) is capable of synthesizing a nucleic acid fragment without a template and (ii) is capable of incorporating a 3'-O-modified nucleotide onto a free 3'-hydroxyl of a nucleic acid fragment.

2. The TdT variant of claim 1 wherein:

said substitution for leucine at position 61 in SEQ ID NO: 2, 8 or 11 or position 48 in SEQ ID NO: 5 is selected from the group consisting of R or Q;

5       said substitution for cysteine at position 170 in SEQ ID NO: 2 or position 158 in SEQ ID NO: 5, 29 or 44 or position 171 in SEQ ID NO: 32, 35, 38, 41 or 47 is selected from the group consisting of G, R, P, A, V, S, N, Q or D; and in other embodiments, the substitution for cysteine at the foregoing positions is selected from the group consisting of G or R;

10       said substitution for tyrosine at position 171 of SEQ ID NO: 8 or 11 is selected from the group consisting of G, R, P, A, V, S, N, Q or D; and in other embodiments, the substitution for tyrosine at the foregoing positions is selected from the group consisting of G or R;

      said substitution for alanine at position 158 of SEQ ID NO: 17 or position 171 of SEQ ID NO: 23 or 26 is selected from the group consisting of G, R, P, V, S, N, Q or D; and in other embodiments, the substitution for alanine at the foregoing positions is selected from the group consisting of G or R;

15       said substitution for proline at position 171 of SEQ ID NO: 20 is selected from the group consisting of G, R, A, V, S, N, Q or D; and in other embodiments, the substitution for tyrosine at the foregoing positions is selected from the group consisting of G or R;

      said substitution for arginine at position 204 of SEQ ID NO: 2 or position 192 of SEQ ID NO: 5, 17, 29 or 44 or position 205 of SEQ ID NO: 8, 11, 14, 20, 23, 26, 32, 35, 38, 41 or 20   47 is selected from the group consisting of L or N;

      said substitution for arginine at position 326 of SEQ ID NO: 2 or position 314 of SEQ ID NO: 5 or position 327 of SEQ ID NO: 8 or 14 or position 324 of SEQ ID NO: 11 or position 311 of SEQ ID NO: 17 or position 321 of SEQ ID NO: 20 or position 322 of SEQ ID NO: 23 or 26 or position 310 of SEQ ID NO: 29 or position 323 of SEQ ID NO: 32, 35, 38, 41 25   or 47 or position 309 of SEQ ID NO: 44 is selected from the group consisting of P, N or A;

      said substitution for threonine at position 321 of SEQ ID NO: 20 is selected from the group consisting of P, N or A;

      said substitution for glycine at position 329 of SEQ ID NO: 2 or 5 is selected from the group consisting of N, L, T or S; and

30       said substitution for glutamic acid at position 330 of SEQ ID NO: 8 or 14 or position 327 of SEQ ID NO: 11 or position 311 of SEQ ID NO: 17 or position 324 of SEQ ID NO: 20

or position 325 of SEQ ID NO: 23 or 26 or position 313 of SEQ ID NO: 29 position 326 of SEQ ID NO: 32, 35, 38, 41 or 47 or position 312 of SEQ ID NO: 44 is selected from the group consisting of N, L, T or S.

- 5     3. The TdT variant of claim 1 or 2 wherein:  
with respect to SEQ ID NO: 2 glutamine at position 327 is substituted;  
with respect to SEQ ID NO: 5 glutamic acid at position 315 is substituted;  
with respect to SEQ ID NO: 8 glutamine at position 328 is substituted;  
with respect to SEQ ID NO: 11 glutamine at position 325 is substituted;  
10    with respect to SEQ ID NO: 14 glutamine at position 328 is substituted;  
with respect to SEQ ID NO: 17 glutamine at position 312 is substituted;  
with respect to SEQ ID NO: 20 glutamine at position 322 is substituted;  
with respect to SEQ ID NO: 23 glutamine at position 323 is substituted;  
with respect to SEQ ID NO: 26 methionine at position 323 is substituted;  
15    with respect to SEQ ID NO: 29 glutamine at position 311 is substituted;  
with respect to SEQ ID NO: 32 glutamine at position 324 is substituted;  
with respect to SEQ ID NO: 35 glutamine at position 324 is substituted;  
with respect to SEQ ID NO: 38 glutamine at position 324 is substituted;  
with respect to SEQ ID NO: 41 glutamine at position 324 is substituted;  
20    with respect to SEQ ID NO: 44 glutamine at position 310 is substituted; and  
with respect to SEQ ID NO: 47 glutamine at position 324 is substituted.
4. The TdT variant of claim 3 wherein said glutamine is substituted with an amino acid selected from the group consisting of T, F, L, M, I, V and Y.
- 25    5. The TdT variant of any of claims 1, 2, 3 or 4 wherein said 3'-O-modified nucleotide is selected from the group consisting of 3'-O-amino-2'-deoxynucleoside triphosphate, 3'-O-azidomethyl-2'-deoxynucleoside triphosphate and 3'-O-allyl-2'-deoxynucleoside triphosphate.

6. A terminal deoxynucleotidyl transferase (TdT) variant comprising an amino acid sequence at least ninety percent identical to an amino acid sequence selected from SEQ ID NO: 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36, 39, 42, 45 or 48, wherein:

with respect to SEQ ID NO: 3 amino acid position 61 is R, amino acid position 171 is  
5 R, amino acid position 205 is L, amino acid position 327 is A, and amino acid position 330 is N;

with respect to SEQ ID NO: 6 amino acid position 48 is R, amino acid position 158 is R, amino acid position 192 is L, amino acid position 314 is P, and amino acid position 317 is N;

10 with respect to SEQ ID NO: 9 amino acid position 61 is R, amino acid position 171 is R, amino acid position 205 is L, amino acid position 327 is A, and amino acid position 330 is N;

with respect to SEQ ID NO: 12 amino acid position 61 is R, amino acid position 171 is A, amino acid position 205 is L, amino acid position 324 is P, and amino acid position 327 is  
15 N;

with respect to SEQ ID NO: 15 amino acid position 61 is R, amino acid position 171 is R, amino acid position 205 is L, amino acid position 328 is P, and amino acid position 331 is N;

with respect to SEQ ID NO: 18 amino acid position 48 is R, amino acid position 158 is  
20 R, amino acid position 192 is L, amino acid position 311 is P, and amino acid position 314 is N;

with respect to SEQ ID NO: 21 amino acid position 61 is R, amino acid position 171 is P, amino acid position 205 is L, amino acid position 321 is A, and amino acid position 324 is N;

25 with respect to SEQ ID NO: 24 amino acid position 61 is R, amino acid position 171 is A, amino acid position 205 is L, amino acid position 322 is A, and amino acid position 325 is N;

with respect to SEQ ID NO: 27 amino acid position 61 is R, amino acid position 171 is R, amino acid position 205 is L, amino acid position 327 is A, and amino acid position 330 is  
30 N;

with respect to SEQ ID NO: 30 amino acid position 48 is R, amino acid position 158 is R, amino acid position 192 is L, amino acid position 310 is P, and amino acid position 313 is N;

5 with respect to SEQ ID NO: 33 amino acid position 61 is R, amino acid position 171 is R, amino acid position 205 is L, amino acid position 323 is P, and amino acid position 326 is N;

with respect to SEQ ID NO: 36 amino acid position 61 is R, amino acid position 171 is R, amino acid position 205 is L, amino acid position 323 is A, and amino acid position 326 is N;

10 with respect to SEQ ID NO: 39 amino acid position 61 is R, amino acid position 171 is R, amino acid position 205 is L, amino acid position 323 is P, and amino acid position 326 is N;

with respect to SEQ ID NO: 42 amino acid position 61 is R, amino acid position 171 is R, amino acid position 205 is L, amino acid position 323 is A, and amino acid position 326 is N;

15 with respect to SEQ ID NO: 45 amino acid position 48 is R, amino acid position 158 is R, amino acid position 192 is L, amino acid position 310 is A, and amino acid position 313 is N;

20 with respect to SEQ ID NO: 48 amino acid position 61 is R, amino acid position 171 is R, amino acid position 205 is L, amino acid position 323 is P, and amino acid position 326 is N; and

wherein the TdT variant (i) is capable of synthesizing a nucleic acid fragment without a template and (ii) is capable of incorporating a 3'-O-modified nucleotide onto a free 3'-hydroxyl of a nucleic acid fragment.

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7. The TdT variant of claim 6 wherein:

with respect to SEQ ID NO: 3 amino acid position 328 is Q or is selected from the group consisting of T, F, L or M;

30 with respect to SEQ ID NO: 6 amino acid position 315 is Q or is selected from the group consisting of T, F, L or M;

with respect to SEQ ID NO: 9 amino acid position 328 is Q or is selected from the group consisting of T, F, L or M;

with respect to SEQ ID NO: 12 amino acid position 325 is Q or is selected from the group consisting of T, F, L or M;

5       with respect to SEQ ID NO: 15 amino acid position 329 is Q or is selected from the group consisting of T, F, L or M;

with respect to SEQ ID NO: 18 amino acid position 312 is Q or is selected from the group consisting of T, F, L or M;

10       with respect to SEQ ID NO: 21 amino acid position 322 is Q or is selected from the group consisting of T, F, L or M;

with respect to SEQ ID NO: 24 amino acid position 323 is Q or is selected from the group consisting of T, F, L or M;

with respect to SEQ ID NO: 27 amino acid position 328 is Q or is selected from the group consisting of T, F, L or M;

15       with respect to SEQ ID NO: 30 amino acid position 311 is Q or is selected from the group consisting of T, F, L or M;

with respect to SEQ ID NO: 33 amino acid position 324 is Q or is selected from the group consisting of T, F, L or M;

20       with respect to SEQ ID NO: 36 amino acid position 324 is Q or is selected from the group consisting of T, F, L or M;

with respect to SEQ ID NO: 39 amino acid position 324 is Q or is selected from the group consisting of T, F, L or M;

with respect to SEQ ID NO: 42 amino acid position 324 is Q or is selected from the group consisting of T, F, L or M;

25       with respect to SEQ ID NO: 45 amino acid position 311 is Q or is selected from the group consisting of T, F, L or M; and

with respect to SEQ ID NO: 48 amino acid position 324 is Q or is selected from the group consisting of T, F, L or M.

8. The TdT variant of any of claims 6 or 7 wherein said 3'-O-modified nucleotide is selected from the group consisting of 3'-O-amino-2'-deoxynucleoside triphosphate, 3'-O-azidomethyl-2'-deoxynucleoside triphosphate and 3'-O-allyl-2'-deoxynucleoside triphosphate.

- 5 9. A terminal deoxynucleotidyl transferase (TdT) variant comprising an amino acid sequence at least ninety percent identical to an amino acid sequence selected from SEQ ID NO: 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36, 39, 42, 45 or 48, and wherein the TdT variant (i) is capable of synthesizing a nucleic acid fragment without a template and (ii) is capable of incorporating a 3'-O-modified nucleotide onto a free 3'-hydroxyl of a polynucleotide.

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10. A method of synthesizing a polynucleotide having a predetermined sequence, the method comprising the steps of:

a) providing an initiator having a 3'-terminal nucleotide having a free 3'-hydroxyl;

- 15 b) repeating cycles of (i) contacting under elongation conditions the initiator or elongated fragments having free 3'-O-hydroxyls with a 3'-O-blocked nucleoside triphosphate and a TdT variant according to any one of claims 1 to 9, so that the initiator or elongated fragments are elongated by incorporation of a 3'-O-blocked nucleoside triphosphate to form 3'-O-blocked elongated fragments, and (ii) deblocking the elongated fragments to form elongated fragments having free 3'-hydroxyls, until the polynucleotide is formed.

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11. The method of claim 10 wherein said 3'-O-blocked nucleoside triphosphate is a 3'-O-NH<sub>2</sub>-nucleoside triphosphate, a 3'-O-azidomethyl-nucleoside triphosphate, or a 3'-O-allyl-nucleoside triphosphate.

- 25 12. A kit for performing a nucleotide incorporation reaction comprising:

a) a TdT variant according to any one of claims 1 to 9, b) one or more 3'-O-protected nucleoside triphosphates, and c) optionally at least one initiator.