Assay Development Proof of Concept I

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1 Procedure Purpose

Perform a full validation of whether the technique employed by the assay development team is capable of testing backspace synthesis. Test every step in the assay to troubleshoot any problematic components. Determine whether a 5-bp or a 6-bp complementary strand will lead to more efficient nucleotide addition. Create positive and negative control results for future comparison.

2 Overview

In order to conduct DNA synthesis using water-soluble and non-toxic materials, a method using a pair of enzymes to extend and shorten a pre-existing template strand has been devised and termed the 'backspace' method. This technique involves three steps:

1. Elongating an existing single-stranded DNA strand with an arbitrary length of one nucleotide of choice using Terminal Deoxynucleotidyl Transferase (TdT).
2. Adding a complementary strand that matches the 3' end of the original starting sequence, as well as one or two of the added nucleotides.
3. Introducing Exonuclease T, which will cleave the 3' region of the elongated starting strand, effectively cleaving excess nucleotides that are not to be added. [1]

This experiment is intended to implement each of the steps of the 'backspace' technique, resulting in the addition of a single nucleotide to a pre-determined ssDNA strand. This is the first test of the AD assay, in which addition onto a pre-determined 350bp strand is assayed using Sanger sequencing. The AD assay consists of the following:

1. Creating a small 4-bp overhang on a template strand by using the BstXI restriction enzyme.
2. Performing backspace synthesis, as described above, on the template strand.
3. Poly-A tailing the template strand.
4. Creating a fully double-stranded sequence by filling in the single-stranded region using a Taq polymerase and an oligo d(T)18 primer.
5. Sanger sequencing the resulting strand. This experiment will also create positive and negative controls in order to better determine successful addition using the backspace synthesis method.

3 Safety Information

1. **SYBR Gold** has no data available addressing the mutagenicity or toxicity of SYBR® Gold nucleic acid gel stain. Because this reagent binds to nucleic acids, it should be treated as a potential mutagen and handled with appropriate care. The DMSO stock solution should be handled with particular caution as DMSO is known to facilitate the entry of organic molecules into tissues.[2]

4 Sample Explanations

All samples will have R1-4 following them, indicating which replicate they belong to. Each sample and control will have four replicates.

1. **AD5**, Assay Development 5 - Sample testing the entirety of the backspace synthesis method using a complementary strand consisting of five basepairs.
2. **AD5**, Assay Development 5 - Sample testing the entirety of the backspace synthesis method using a complementary strand consisting of six basepairs.
3. **ADNC**, Assay Development Negative Control - Control designed to generate Sanger sequencing reads without backspace synthesis.
4. **ADPC**, Assay Development Positive Control - Control designed to generate Sanger sequencing reads designed to imitate successful single basepair addition.

5 Materials

- BstXI-Digested, Verified AD Template
- Verified AD Positive Control
- 100μM Dev_Seq_Fwd2 Primer
- 100μM Dev_Comp_5_Pure Primer
- 100μM Dev_Comp_6_Pure Primer
- Agarose
- Nuclease-free Water
- 1X Tris-Borate-EDTA (TBE) Buffer
- 10,000X SYBR Green I
- Thermocycler
- 30U/μL Terminal Deoxynucleotidyl Transferase
- 5X TdT Buffer
- Exonuclease T
- NEBuffer 4
- EDTA
- Qiagen PCR Purification Kit
- 100mM dATP
- 100mM dTTP
- 10mM dNTPs
- 10X Taq Reaction Buffer Pack
- Oligo d(T)18 Primer
- PCR 8-strip Tubes

6 Procedure

6.1 TdT Poly-T Tailing

1. Dilute verified and purified BstXI-digested template DNA to a concentration of 25ng/μL.
2. Label twelve tubes as per the following:
   (a) AD5 R1 - AD5 R4
   (b) AD6 R1 - AD6 R4
   (c) ADNC R1 - ADNC R4
3. Into each tube, pipette the following:
   (a) 18.5μL nuclease-free water.
   (b) 20μL template DNA
   (c) 10μL 5X TdT Buffer.
   (d) 1μL 100mM dTTP.
   (e) .5μL 30U/μL Terminal Deoxynucleotidyl Transferase
4. Incubate at 37°C for an hour.
5. Stop the reaction by heating at 70°C for ten minutes.

OPTIONAL STOP POINT

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6. Run 2μL of the reaction on a 2% agarose gel. Ensure that strands are uniformly tailed and that there is not a significant portion of template that is not tailed. If tailing is not complete, proceed to the next step and then repeat TdT tailing.
7. Purify the reaction using a Qiagen PCR Purification kit.

OPTIONAL STOP POINT

6.2 Exonuclease T Cleavage

6.3 Positive Control A-Overhang Addition

6.4 TdT Poly-A Tailing

6.5 Complementary Strand Fill-In

7 Analysis

8 Conclusions

References

[1] Y. Zuo and M. P. Deutscher, "The DNase activity of RNase T and its application to DNA cloning," *Nucleic Acids Research*, vol. 27, no. 20, p. 4077–4082, 1999.

[2] Invitrogen, "SYBR® Gold Nucleic Acid Gel Stain — 2 Working with the SYBR® Gold Gel Stain,"