3’-O-(2-nitrobenzyl)-2’-dATP Incorporation Detection with PAGE Assisted Precision Version 5

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1 Procedure Purpose

Determine if the the modified nucleotide, 3’-O-(2-nitrobenzyl)-2’-dATP, can be noticeably incorporated by Terminal Deoxynucleotidyl Transferase in "standard conditions" while determining the blocking efficacy & purity of our 3’-O-(2-nitrobenzyl)-2’-dATP stock.

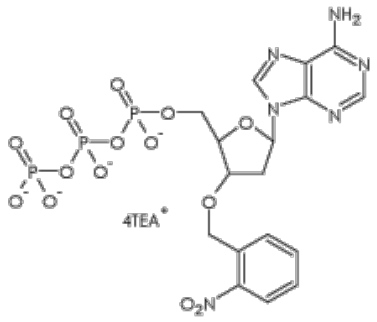

Figure 1: 3’-O-(2-nitrobenzyl)-2’-dATP

2 Overview

This lab will attempt to append 3’-O-(2-nitrobenzyl)-2’-dATP to a **short (25bp) primer**. The effectiveness of this attempt will be determined by attempting to form a homopolymer on the modified primer. If a homopolymer is formed, the blocking groups did not effectively prevent their formation. This could be due to many reasons (the most likely of which being that the blocking groups either (1) were appended without the 2’ nitrobenzyl due to sample degradation or (2) were not appended). If the homopolymer was not formed (but a homopolymer was formed on the controls) it follows that the blocking groups prevented the formation of the homopolymer, likely due to them preforming their intended function. Moreover, all samples will be run on a PAGE gel in order to achieve single nucleotide resolution. This will allow us to confirm that the 3’-O-(2-nitrobenzyl)-2’-dATP is the only base appended to the "blocked" sample. A ddATP control will help as well.

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.[1]
2. **Terminal Deoxynucleotidyl Transferase** is toxic if inhaled. May cause cancer. Toxic to aquatic life with long lasting effects. Avoid breathing dust/fume/gas/mist/vapours/spray. Use personal protective equipment as required. If Inhaled: Remove victim to fresh air and keep at rest in a position comfortable for breathing. Dispose of contents/container in accordance with local/regional/national/international regulations.[2]
3. Working in a communal lab space is dangerous. Do not assume your fellow workers cleaned up sufficiently

4 Materials

- Primer (25bp)
- 100mM 3'-O-(2-nitrobenzyl)-2'-dATP Stock
- 10mM dNTP Stock
- 100mM dATP Stock
- 10mM ddATP Stock
- 5X Terminal Deoxynucleotidyl Transferase Buffer
- Terminal Deoxynucleotidyl Transferase Stock (20U/μL)
- Nuclease Free Water
- TBE Buffer
- 20% Urea Denaturing Gels
- SYBR Gold

5 Procedure

5.1 Sample Preparation

A	The primer incubated with **dATP** and then commercial **dNTPs**
B1	The primer incubated with *just* **NBdATP**
B2	The primer incubated with **NBdATP** and then **dNTPs**
C	The primer incubated with just **dNTPs** in the second incubation
D	The primer incubated with **ddATP** nucleotides and then **dNTPs**
X	The primer incubated with **dNTPs** but **no Terminal Deoxynucleotidyl Transferase** in the second incubation

Figure 2: Samples and their experimental conditions

1. Remove 3'-O-(2-nitrobenzyl)-2'-dATP, Terminal Deoxynucleotidyl Transferase, primer, Terminal Deoxynucleotidyl Transferase buffer, ddATP stock and dATP stock from -20°C freezer

2. Let 3'-O-(2-nitrobenzyl)-2'-dATP thaw on ice in dark
3. Other reagents can thaw on ice in the light

5.2 Attempted blocking

4. Label three PCR Tubes A, B and D, respectively
5. Pipette 11µL of nuclease free water into tube A
6. Pipette 13.7µL of nuclease free water into tube B
7. Pipette 11µL of nuclease free water into tube D
8. Pipette 4.0µL 5X Terminal Deoxynucleotidyl Transferase reaction buffer into all three PCR Tubes
9. Dilute Nucleotides:
   (a) Label a PCR Tube "dATP Dilute"
   (b) Pipette 9µL of nuclease free water into PCR Tube
   (c) Pipette 1µL of dATP stock into PCR Tube
   (d) Vortex directly before use
10. Pipette 0.5µL of primer into all three PCR Tubes
11. Pipette 3µL of dATP dilute into PCR Tube A
12. Pipette .3µL of 3'-O-(2-nitrobenzyl)-2'-dATP stock into PCR Tube B
13. Pipette 3µL of ddATP **10mM stock** into PCR Tube D
14. Gently pipette 1.5µL Terminal Deoxynucleotidyl Transferase(20U/µL) into both PCR tubes.
15. Incubate sample at 37°C for 30 minutes
    Note: **Do NOT** deactivate Terminal Deoxynucleotidyl Transferase

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OPTIONAL STOP POINT

5.3 Extending

Based off of our standard Terminal Deoxynucleotidyl Transferase extending procedure [3].

16. Label two PCR Tubes C and X, respectively
17. Pipette 14µL nuclease free water into PCR Tube C (see above, **ATTEMPTED BLOCKING**)
18. Pipette 15.5µL of nuclease free water into PCR Tube X (see above, **CONTROLS**)
19. Pipette 0.5µL of primer into both PCR Tubes
20. Set PCR Tube X aside.
21. Pipette 4.0µL 5X Terminal Deoxynucleotidyl Transferase reaction buffer into PCR Tube C
22. Pipette 3µL of dNTP stock into PCR Tubes C and X
23. Pipette .4µL of dNTP stock into PCR Tubes **A, B**
24. Wait until the previous samples have finished incubating

25. Label a PCR Tube "B1"
26. Pipette 10µL from B into B1
27. Pipette 1.5µL EDTA into **B1** to stop the reaction [2]
28. Gently pipette 1.5µL Terminal Deoxynucleotidyl Transferase (15 U/µL) into **PCR Tube C**
29. Incubate **all** samples **except B1** at 37°C for 30 minutes
30. Pipette 1.5µL nuclease free water into B1
31. Place B1 into -20°C freezer for later use
32. Stop any Terminal Deoxynucleotidyl Transferaseaction by adding 2µL 0.5M EDTA to the all PCR tubes **except B2** after incubation.[2]
33. Pipette 1µL 0.5M EDTA into B2

RECOMMENDED STOP POINT

5.4 Analysis

5.5 XCell Surelock Setup and Pre-Run

34. Remove 20% polyacrylamide gel from pouch and rinse with deionized water.
35. Peel off tape on bottom of 20% polyacrylamide gel and remove the comb.
36. Gently wash every cassette well with 1X TBE buffer. Invert to remove buffer and shake. Repeat twice.
37. Lower the Buffer Core (the piece that holds the gels) into the Lower Buffer Chamber so that the negative electrode fits into the opening in the gold plate.
38. Insert the Gel Tension Wedge into the XCell Surelock behind the buffer core. Make sure it is in its 'unlocked' position, which allows the wedge to slip into the unit.
39. Insert gel cassettes into the lower buffer chamber. The shorter "well" side of the cassette faces into the buffer core. The slot on the back must face outward. If only one gel is being run, insert a buffer dam in the place of a gel cassette.
40. Pull forward on the Gel Tension Lever toward the buffer core until the gel cassettes are snug against the buffer core. This puts it in the 'locked' position.
41. Fill the Upper Buffer Chamber (between the gels) with running buffer. Ensure it is not leaking.
42. Fill the Lower Buffer Chamber completely with running buffer by pouring TBE next to the Gel Tension Wedge.
43. Pipette 12µL of running buffer into each gel well.
44. Place the gel cover on the apparatus in the correct orientation. Connect the electrodes to the power source, and pre-run the gel for 30 minutes at 150V.
45. When there is only 5 minutes left on the incubation, retreive sample B1 from the freezer and let thaw on ice

Well number	Sample
1	10/60 DNA Ladder
2	Custom Ladder (40ng)
3	B1 (40ng)
4	B2 (40ng)
5	X (40ng)
6	X + B1 (40ng each)
7	X + B2 (40ng each)
8	D (40ng)
9	X + D (40ng each)
10	A (40ng)
11	C (40ng)
12	26-Mer (40ng)
13	25-Mer (40ng)
14	Custom Ladder (40ng)
15	10/60 DNA Ladder

Figure 3: Wells

5.5.1 Run Gel

Note: Be relatively swift about mixing and loading, as the samples will gradually begin to evaporate if left on the parafilm for too long.

46. Obtain a sizable piece of parafilm. Pipette 3 µL of Gel Loading Buffer in a row of 15 droplets.
47. For the 10/60 Ladder samples, pipette 1uL of 10/60 Ladder and 4 uL of running buffer and mix.
48. For the remaining droplets, add 3 µL of the appropriate sample. See the corresponding table (Figure 5.5.1) above for sample location and order.
49. As you go, pipette up and down to mix thoroughly.
50. Load the gels (with 5 µL sample in each well) when they are finished pre-running. Ensure pipette tip is fully in the well, and depress slowly and carefully. Work quickly to minimize diffusion.
51. Run the gel(s) at 150V until the dark blue dye is at the bottom.

5.5.2 Stain & View Gel

52. While the gel runs, prepare 1X SYBR Gold Staining Solution with TBE as dilute
    (a) Add 6µL SYBR Gold to 60µL of TBE running buffer
53. Once gel has finished running, ***lightly*** agitate gel while submerged in solution for 60 minutes.
54. Review gel with gel viewer. Until unnecessary, place gel back in stain for 20 minute increments and re-image.
55. Post pictures to Slack.

Stop Procedure

1. Pipette samples into PCR tubes if not already contained in an appropriate manner
2. Label containers if not already labeled
3. Freeze samples at -20°C

References

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

[2] Invitrogen, "Terminal Deoxynucleotidyl Transferase, Recombinant Technical Bulletin 8008-1," tech. rep., 2002.

[3] M. Uttmark, S. Gurev, M. Arcidiacono, A. Tomusiak, C. Hao, and R. Fuize, "Generic Terminal Deoxynucleotidyl Transferase Extending Procedure," 2017.