3'-O-(2-nitrobenzyl)-2'-dATP Incorporation and Removal Charecterization with PAGE Assisted Precision Version 1

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1 Procedure Purpose

Determine if the modified nucleotide, 3'-O-(2-nitrobenzyl)-2'-dATP, can be noticeably incorporated and then removed by Terminal Deoxynucleotidyl Transferase in "standard conditions".

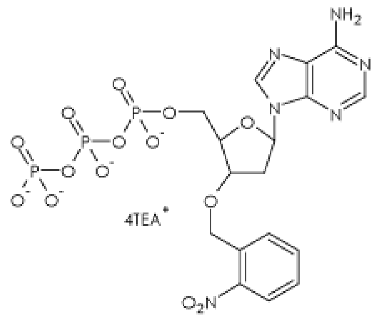

Figure 1: 3'-O-(2-nitrobenzyl)-2'-dATP

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. Moreover, another sample will have their blocking groups removed by ultraviolet light (365nm) and the will be treated with the same dNTP extension process as the "blocked" sample. 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 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 determine the effectiveness of the blocking group as a positive control for effective blocking.

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. **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.[**?**]
3. **Terminal Deoxynucleotidyl Transferase Buffer** may cause cancer. It is also a skin irritant. However, ingestion/inhalation is not expected to present a significant ingestion hazard under anticipated conditions of normal use. If you feel unwell, seek medical advice.
4. 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
- 365nm UV Radiation Source (We will be using our "UV Death Chamber")

5 Procedure

5.1 Sample Preparation

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

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**
B3	The primer incubated with **NBdATP**, irradiated with 365nm ultraviolet light, and then **dNTPs**
B1*, B2* B3*	A repeat of B1, B2 and B3 prepared separately from B1, B2 and B3
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

Figure 2: Samples and their experimental conditions

5.2 Attempted blocking

4. Label four PCR Tubes A, B, B* and D, respectively
5. Pipette 11μL of nuclease free water into tube A
6. Pipette 20.55μL of nuclease free water into tube B and B*
7. Pipette 11μL of nuclease free water into tube D
8. Pipette 4.0μL 5X Terminal Deoxynucleotidyl Transferase reaction buffer into PCR Tubes A and D
9. Pipette 6.0μL 5X Terminal Deoxynucleotidyl Transferase reaction buffer into PCR Tube B and B*
10. 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
11. Pipette 0.5μL of primer into PCR Tubes A and D
12. Pipette 0.75μL of primer into B and B*
13. Pipette 3μL of dATP dilute into PCR Tube A
14. Pipette 0.45μL of 3'-O-(2-nitrobenzyl)-2'-dATP stock into PCR Tube B and B*
15. Pipette 3μL of ddATP **10mM stock** into PCR Tube D
16. Gently pipette 1.5μL Terminal Deoxynucleotidyl Transferase(20U/μL) into PCR tubes A and D.
17. Gently pipette 2.25μL Terminal Deoxynucleotidyl Transferase(20U/μL) into PCR tube B and B*.
18. Incubate samples at 37°C for 30 minutes
19. Return dATP and ddATP to -20°C freezer.

OPTIONAL STOP POINT

5.3 Extending

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

20. Label two PCR Tubes C and X, respectively
21. Pipette 14μL nuclease free water into PCR Tube C (see above, **ATTEMPTED BLOCKING**)
22. Pipette 15.5μL of nuclease free water into PCR Tube X (see above, **CONTROLS**)
23. Pipette 0.5μL of primer into both PCR Tubes
24. Set PCR Tube X aside.
25. Pipette 4.0μL 5X Terminal Deoxynucleotidyl Transferase reaction buffer into PCR Tube C
26. Pipette 3μL of dNTP stock into PCR Tubes C and X
27. Wait until the previous samples have finished incubating
28. Label two PCR Tubes "B1" and "B1*
29. Label two PCR Tubes "B3" and "B3*
30. Relabel PCR Tubes B and B* as B2 and B2* respectively
31. Pipette 10μL from B2 into B1
32. Pipette 10μL from B2* into B1*
33. Pipette 10μL from B2 into B3
34. Pipette 10μL from B2* into B3*
35. Pipette 1.5μL EDTA into **B1 and B1*** to stop the reaction [?]

36. Pipette 1.5μL nuclease free water into B1 and B1*
37. Place B1 and B1* into -20°C freezer for later use
38. Expose B3 and B3* to 365nm of ultraviolet light for 15 minutes with UV deathchamber, or 30 minutes with flashlight if chamber is not available
39. Pipette .4μL of dNTP stock into PCR Tubes **A**
40. Pipette .4μL of dNTP stock into PCR Tubes **B2**
41. Pipette .4μL of dNTP stock into PCR Tubes **B3**
42. Pipette .4μL of dNTP stock into PCR Tubes **B2***
43. Pipette .4μL of dNTP stock into PCR Tubes **B3***
    Please check these off as you go - it's easy to miss one.
44. Gently pipette 1.5μL Terminal Deoxynucleotidyl Transferase (20 U/μL) into **PCR Tube C** and **B3** along with **B3***
45. Incubate **all** samples **except B1 and B1*** at 37°C for 30 minutes
46. Wait until the samples have finished incubating.
47. Stop any Terminal Deoxynucleotidyl Transferaseaction by adding 2μL 0.5M EDTA to the all PCR tubes **except B and B*** after incubation.[?]
48. Pipette 1μL 0.5M EDTA into B2 and B2*

RECOMMENDED STOP POINT

5.4 Analysis

5.4.1 XCell Surelock Setup and Pre-Run

49. Remove 20% polyacrylamide gel from pouch and rinse with deionized water.

50. Peel off tape on bottom of 20% polyacrylamide gel and remove the comb.

51. 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.

52. 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.

53. 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.

54. 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.

55. Fill the Upper Buffer Chamber (between the gels) with running buffer. Ensure it is not leaking.

56. Fill the Lower Buffer Chamber completely with running buffer by pouring TBE next to the Gel Tension Wedge.

57. Pipette 12μL of running buffer into each gel well.

58. 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.

59. When there is only 5 minutes left on the incubation, retrieve sample B1 and B1* from the freezer and let thaw on ice

5.4.2 Run Gel

Well number	Sample
1	10/60 DNA Ladder
2	Custom Ladder (40ng)
3	B1 (40ng)
4	B2 (40ng)
5	B3 (40ng)
6	B1* (40ng)
7	B2* (40ng)
8	B3* (40ng)
9	X (40ng)
10	D (40ng)
11	X + D (40ng each)
12	A (40ng)
13	C (40ng)
14	Custom Ladder (40ng)
15	10/60 DNA Ladder

Figure 3: Wells and their assorted reagents

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

60. Obtain a sizable piece of parafilm. Pipette 3 μL of 2X Gel Loading Dye in a row of 15 droplets.
61. For the 10/60 Ladder samples, pipette 1uL of 10/60 Ladder and 4 uL of running buffer and mix.
62. For the remaining droplets, add 3 μL of the appropriate sample. See the corresponding table (Figure 5.4.2) above for sample location and order.
63. As you go, pipette up and down to mix thoroughly.
64. 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.
65. Run the gel(s) at 150V until the dark blue dye is at the bottom.

5.4.3 Stain & View Gel

66. 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
67. Once gel has finished running, ***lightly*** agitate gel while submerged in solution for 60 minutes.
68. Review gel with gel viewer. Until unnecessary, place gel back in stain for 20-minute increments and re-image.
69. 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