

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

Written by **Michael Uttmark**
For the Stanford Student Space Initiative Biology Sub team

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1 Procedure Purpose

Determine if 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.

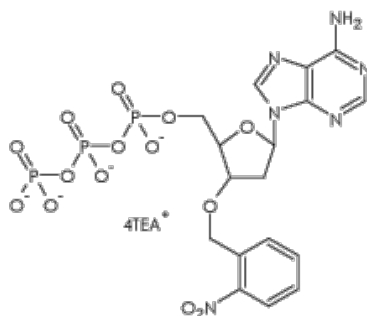


Figure 1: 3'-O-(2-nitrobenzyl)-2'-dATP

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 performing 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 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. **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/ μ L)
- Nuclease Free Water
- TBE Buffer
- 20% Urea Denaturing Gels
- SYBR Gold

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 <i>just</i> NBdATP
B2	The primer incubated with NBdATP and then dNTPs
B1*, B2*	A repeat of B1 and B2 prepared separately from B1 and B2
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

5.2 Attempted blocking

4. Label four PCR Tubes A, B, B* and D, respectively
5. Pipette 11 μ L of nuclease free water into tube A
6. Pipette 13.7 μ L of nuclease free water into tube B
7. Pipette 11 μ L of nuclease free water into tube D
Note: Now would be a good time to do step 15.
8. Pipette 4.0 μ L 5X Terminal Deoxynucleotidyl Transferase reaction buffer into all three PCR Tubes
Note: Now would be a good time to do step 16.
9. Dilute Nucleotides:
 - (a) Label a PCR Tube "dATP Dilute"
 - (b) Pipette 9 μ L of nuclease free water into PCR Tube
 - (c) Pipette 1 μ L of dATP stock into PCR Tube
 - (d) Vortex directly before use
10. Pipette 0.5 μ L of primer into all three PCR Tubes
Note: Now would be a good time to do step 17.
11. Pipette 3 μ L of dATP dilute into PCR Tube A
12. Pipette .3 μ L of 3'-O-(2-nitrobenzyl)-2'-dATP stock into PCR Tube B
Note: Now would be a good time to do step 18.
13. Pipette 3 μ L of ddATP **10mM stock** into PCR Tube D
14. Gently pipette 1.5 μ L Terminal Deoxynucleotidyl Transferase(20U/ μ L) into all three PCR tubes.
Note: Now you should do step 19.

5.2.1 Replicate of B: B*

15. Pipette 13.7 μ L of nuclease free water into tube B*
16. Pipette 4.0 μ L 5X Terminal Deoxynucleotidyl Transferase reaction buffer into B*
17. Pipette 0.5 μ L of primer into all three PCR Tubes
18. Pipette .3 μ L of 3'-O-(2-nitrobenzyl)-2'-dATP stock into PCR Tube B*
19. Gently pipette 1.5 μ L Terminal Deoxynucleotidyl Transferase(20U/ μ L) into B*.
20. Incubate samples at 37°C for 30 minutes



21. Return dATP and ddATP to -20°C freezer.

OPTIONAL STOP POINT

5.3 Extending

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

22. Label two PCR Tubes C and X, respectively
23. Pipette 14 μ L nuclease free water into PCR Tube C (see above, **ATTEMPTED BLOCKING**)
24. Pipette 15.5 μ L of nuclease free water into PCR Tube X (see above, **CONTROLS**)
25. Pipette 0.5 μ L of primer into both PCR Tubes
26. Set PCR Tube X aside.
27. Pipette 4.0 μ L 5X Terminal Deoxynucleotidyl Transferase reaction buffer into PCR Tube C
28. Pipette 3 μ L of dNTP stock into PCR Tubes C and X
29. Wait until the previous samples have finished incubating
30. Label two PCR Tubes "B1" and "B1"
31. Pipette 10 μ L from B into B1
32. Pipette 1.5 μ L EDTA into **B1** to stop the reaction [2]
Note: Now would be a good time to do steps 41 and 42.
33. Pipette .4 μ L of dNTP stock into PCR Tubes **A, B**
34. Gently pipette 1.5 μ L Terminal Deoxynucleotidyl Transferase (15 U/ μ L) into **PCR Tube C**
35. Incubate **all** samples **except B1 and B1*** at 37°C for 30 minutes
36. Pipette 1.5 μ L nuclease free water into B1
37. Place B1 into -20°C freezer for later use
Note: Now would be a good time to do steps 43 and 44.
38. Wait until the samples have finished incubating.
39. Stop any Terminal Deoxynucleotidyl Transferase reaction by adding 2 μ L 0.5M EDTA to the all PCR tubes **except B and B*** after incubation.[2]
40. Pipette 1 μ L 0.5M EDTA into B
Note: Now would be a good time to do steps 45 and 46.

5.3.1 Replicates: B1* and B*

41. Pipette 10 μ L from B* into B1*
42. Pipette 1.5 μ L EDTA into **B1*** to stop the reaction [2]
43. Pipette 1.5 μ L nuclease free water into B1*
44. Place B1* into -20°C freezer for later use
45. Pipette 1 μ L 0.5M EDTA into B*



46. Pipette $.4\mu\text{L}$ of dNTP stock into PCR Tube B*

RECOMMENDED STOP POINT

5.4 Analysis

5.4.1 XCell Surelock Setup and Pre-Run

47. Remove 20% polyacrylamide gel from pouch and rinse with deionized water.
48. Peel off tape on bottom of 20% polyacrylamide gel and remove the comb.
49. 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.
50. 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.
51. 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.
52. 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.
53. Fill the Upper Buffer Chamber (between the gels) with running buffer. Ensure it is not leaking.
54. Fill the Lower Buffer Chamber completely with running buffer by pouring TBE next to the Gel Tension Wedge.
55. Pipette $12\mu\text{L}$ of running buffer into each gel well.
56. 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.
57. When there is only 5 minutes left on the incubation, retrieve sample B1 from the freezer and let thaw on ice

5.4.2 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.

58. Obtain a sizable piece of parafilm. Pipette $3\mu\text{L}$ of 2X Gel Loading Dye in a row of 15 droplets.
59. For the 10/60 Ladder samples, pipette $1\mu\text{L}$ of 10/60 Ladder and $4\mu\text{L}$ of running buffer and mix.
60. For the remaining droplets, add $3\mu\text{L}$ of the appropriate sample. See the corresponding table (Figure 5.4.2) above for sample location and order.
61. As you go, pipette up and down to mix thoroughly.
62. Load the gels (with $5\mu\text{L}$ 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.
63. Run the gel(s) at 150V until the dark blue dye is at the bottom.



Well number	Sample
1	10/60 DNA Ladder
2	Custom Ladder (40ng)
3	B1 (40ng)
4	B2 (40ng)
5	B1* (40ng)
6	B2* (40ng)
7	X (40ng)
8	X + B1 (40ng each)
9	X + B2 (40ng each)
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

5.4.3 Stain & View Gel

64. While the gel runs, prepare 1X SYBR Gold Staining Solution with TBE as dilute
 - (a) Add 6 μ L SYBR Gold to 60 μ L of TBE running buffer
65. Once gel has finished running, *lightly* agitate gel while submerged in solution for 60 minutes.
66. Review gel with gel viewer. Until unnecessary, place gel back in stain for 20-minute increments and re-image.
67. 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



6 Analysis

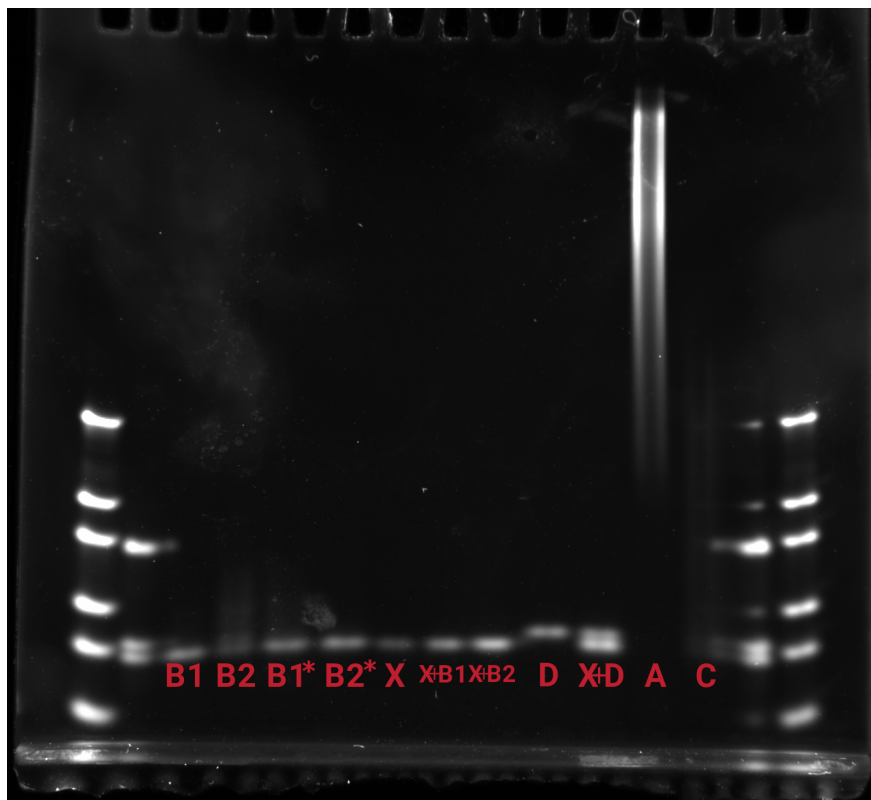


Figure 4: PAGE Gel used to determine length of samples

In this gel, from left to right we have: 10/60 DNA Ladder, Custom Ladder (40ng), B1 (40ng), B2 (40ng), B1* (40ng), B2* (40ng), X (40ng), X+B1 (40ng), X+B2 (40ng), D (40ng), X+D (40ng), A (40ng), C (40ng), Custom Ladder (40ng), and 10/60 DNA Ladder. Upon loading, there was an issue with well 15, leading to contamination of the nearby wells with 10/60 Ladder.

Due to an experimental mistake, B2* was **not** incubated a second time. As such, we only expect the possibility of extension of B2. And, we only see sight extension on B2, none on B1, B1* or B2*. This provides support to the conclusion that 3'-O-(2-nitrobenzyl)-2'-dATP blocks (at the very least) the incorporations of more 3'-O-(2-nitrobenzyl)-2'-dATP. Moreover, there was minimal extension of B2, indicates that the incubation with (and assumed incorporation of) the 3'-O-(2-nitrobenzyl)-2'-dATP hinders (blocks) the incorporation of commercial dNTPs. The main anomaly of this gel is the faintness of sample C. It is only visible (but it is visible) on more exposed images of the gel. In our next experiment, we will likely re-run sample C to check that it has been extended adequately. In short, this experiment supports the claim that 3'-O-(2-nitrobenzyl)-2'-dATP does (in part) block extension.

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.