

3'-O-(2-nitrobenzyl)-2'-dATP Incorporation Detection

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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".

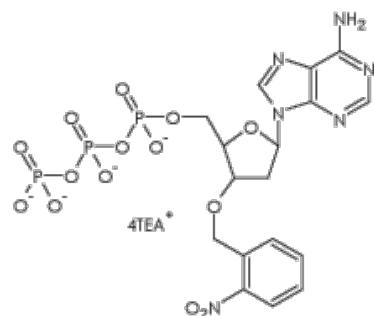
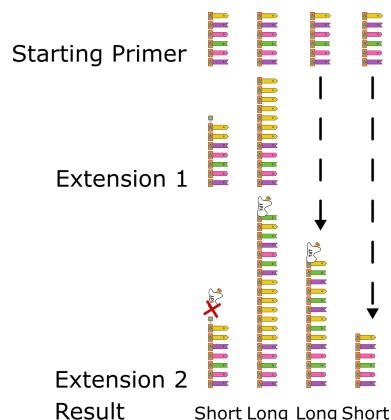


Figure 1: 3'-O-(2-nitrobenzyl)-2'-dATP

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.



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. Working in a communal lab space is dangerous. Do not assume your fellow workers cleaned up sufficiently

4 Materials

- Primer: ATGGACATGCCCTACTTGCATAAG
- 100mM 3'-O-(2-nitrobenzyl)-2'-dATP Stock
- 100mM dNTP Stock
- 100mM dATP Stock
- 5X Terminal Deoxynucleotidyl Transferase Buffer
- Terminal Deoxynucleotidyl Transferase Stock (20U/ μ L)
- Nuclease Free Water
- TBE Buffer
- Agarose
- SYBR Gold

5 Procedure

5.1 Sample Preparation

1. Remove 3'-O-(2-nitrobenzyl)-2'-dATP, Terminal Deoxynucleotidyl Transferase, primer, Terminal Deoxynucleotidyl Transferase buffer and dATP 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 two PCR Tubes A and B, respectively
5. Pipette 10 μ L of nuclease free water into both PCR Tubes
6. Pipette 4.0 μ L 5X Terminal Deoxynucleotidyl Transferase reaction buffer into both PCR Tubes
7. 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 before use
 - (a) Label a PCR Tube "BdATP Dilute" (make this *very* clear)
 - (b) Pipette 9 μ L of nuclease free water into PCR Tube
 - (c) Pipette 1 μ L of 3'-O-(2-nitrobenzyl)-2'-dATP stock into PCR Tube
 - (d) Keep in **dark** and vortex before use
8. Pipette .5 μ L of primer into both PCR Tubes
9. Pipette 2 μ L of dATP dilute into PCR Tube A
10. Pipette 2 μ L of 3'-O-(2-nitrobenzyl)-2'-dATP dilute into PCR Tube B
11. Gently pipette 1.5 μ L Terminal Deoxynucleotidyl Transferase (20 U/ μ L) into both PCR Tubes
12. Incubate sample at 37°C for 30 minutes
 Note: **Do NOT** deactivate Terminal Deoxynucleotidyl Transferase

OPTIONAL STOP POINT

5.3 Extending

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

- 13. Label two PCR Tubes C and X, respectively
- 14. Pipette 10 μ L nuclease free water into PCR Tube C (see above, **ATTEMPTED BLOCKING**)
- 15. Pipette 10 μ L of nuclease free water into PCR Tube X (see above, **CONTROLS**)
- 16. Pipette .5 μ L of primer into both PCR Tubes
- 17. Set PCR Tube X aside.
- 18. Pipette 4.0 μ L 5X Terminal Deoxynucleotidyl Transferase reaction buffer into PCR Tube C
- 19. Pipette .6 μ L of dNTP stock into PCR Tube C
- 20. Pipette .4 μ L of dNTP stock into PCR Tubes **A, B**
- 21. Gently pipette 1.5 μ L Terminal Deoxynucleotidyl Transferase (20 U/ μ L) into **PCR Tube C** (note, this is not from the standard 15U μ L stock)
- 22. Incubate **all** samples at 37°C for 30 minutes

RECOMMENDED STOP POINT

5.4 Analysis

5.4.1 Prepare Gel

We will be preparing a 4% Agarose Gel [?]

- 23. Add 3.2g of Agarose to 80mL of TBE Buffer
- 24. Mix well, and microwave until agarose is completely dissolved
- 25. Pour into gel apparatus and let cool
- 26. Add buffer to gel

5.4.2 Run Gel

27. Add 5 μ L of samples A, B, C and X with 1 μ L of loading dye for a total volume of 5 μ L to gel respectively, left to right with the wells at the top.
28. Add a 50/50 combination of sample **B** and sample C to the sixth well. That is, add 3 μ L of sample **B**, 3 μ L of sample **B** and 1.2 μ L of loading dye to the sixth well, from the left with the wells at the top.
29. Run at 100V (at 1 hour, the voltage was changed to 130V due to time constraints)
30. Remove gel once the Xylene Cyanol FF has almost reached the $\frac{3}{4}$ point [?]

5.4.3 Stain & View Gel

31. While the gel runs, prepare 1X SYBR Gold Staining Solution with TBE as dilute
32. Once gel has finished running, *lightly* agitate gel while submerged in solution for 15 minutes.
33. Review gel with gel viewer. Until unnecessary, place gel back in stain for 10 minute increments and re-image (this was done 4 time on the first run).
34. Post pictures to Slack.
This procedure took 4.5 hours to run.

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 Result Analysis

6.1 Summary

6.2 Gel Analysis

6.4 Procedure Notes

A 4% gel was used, where a 2% gel would have done just as fine. Further, the initial stain should have been 40 minutes, not 15.

List of Figures

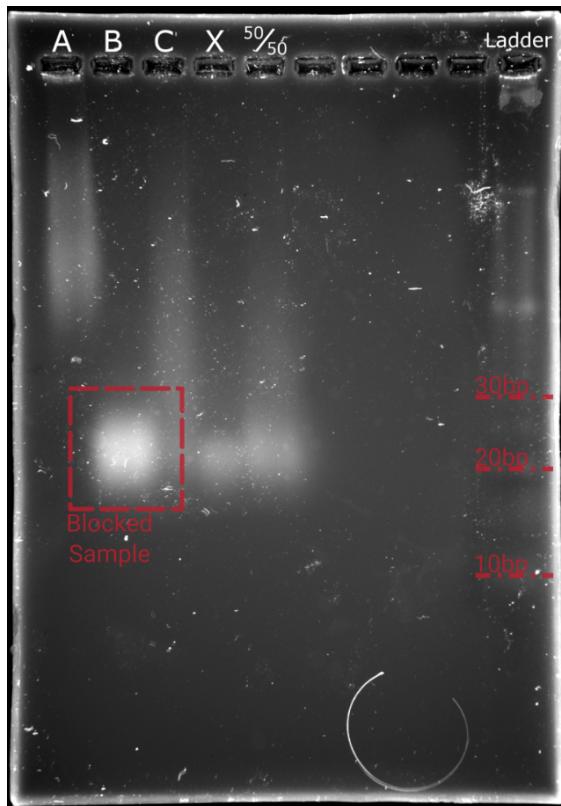


Figure 2: Overlay of two gels, the overnight stain and the 35minute overexposed stain

6.3 Hybrid Gel Analysis

This "gel" is a digital combination of the overnight and 35 minute stain. The gels were combined to reduce diffusion while increasing the visibility of our samples. The unmodified gels are pictured below in figure ??.

In the gel, from left to right, we have sample A (extended control with Adenine homopolymer to test geometric interactions), sample B (the "blocked" sample incubated with the 2'-nitrobenzyl modified adenine), sample C (a standard dNTP extended polymer formed in the second incubation) and sample X (just our 25bp primer) and a 50/50 mix of sample B and sample C. We also have a 10bp ladder from Thermo Fisher in the far right well [?].

Notably, sample B (marked with a dashed box) did not undergo marked extension after being incubated with 3'-O-(2-nitrobenzyl)-2'-dATP. There is some indication of slight extension (predicted due to 3'-O-(2-nitrobenzyl)-2'-dATP degradation). Moreover, the difference between sample A, sample B *and* sample C further supports the conclusion that 3'-O-(2-nitrobenzyl)-2'-dATP was effectively incorporated by Terminal Deoxynucleotidyl Transferase in standard conditions *and* that 3'-O-(2-nitrobenzyl)-2'-dATP effectively prevents further incorporation of nucleotides. However, given that there is only one sample (by the nature of this experiment) that exhibits this behavior, many other factor could have caused this behavior. Most notably, a human error that resulted in the omission of Terminal Deoxynucleotidyl Transferase buffer, dATP, Terminal Deoxynucleotidyl Transferase or nuclease free water would have produced visibly similar results. The fact that samples A and C both underwent successful extensions likely reduces (but does not eliminate) the apparent probability that human error is the source of the apparently promising results. Follow up experiments are necessary for confirmation and characterization.

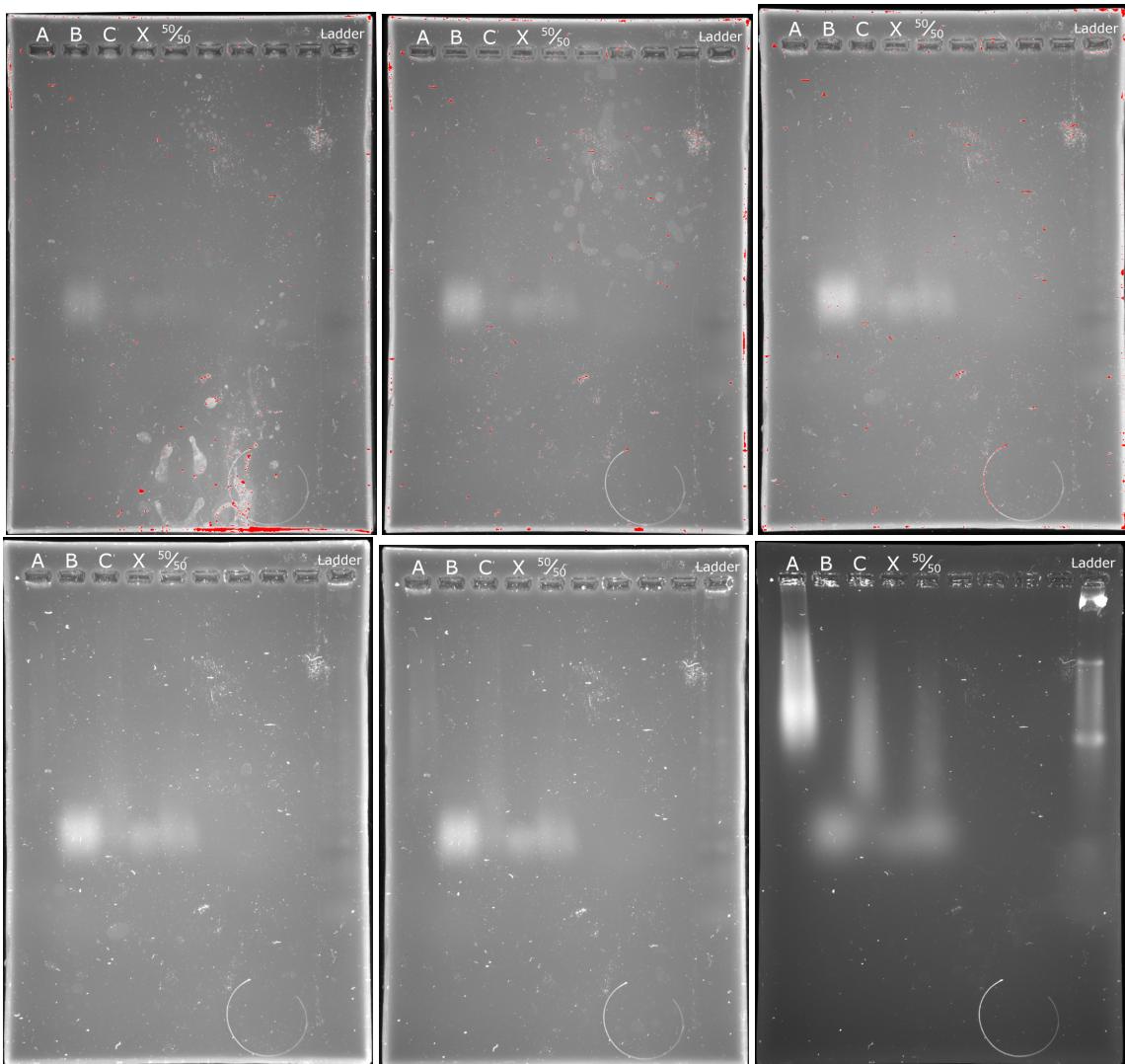


Figure 3: From left to right on the top row, we have 15, 25 and 35 minutes of stain with SYBR Gold all exposed for faint bands. On the bottom from left to right, we have a 2 second exposure of the gel after 35 minutes of stain, a faint bands exposure at 45 minutes of stain and finally a faint bands exposure of the gel after it was stained overnight. All staining was preformed with SYBR Gold.