

3'-O-(2-nitrobenzyl)-2'-dATP Incorporation Detection with PAGE Assisted Precision Version 4: Analysis

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Not Peer Reviewed

For the Stanford Student Space Initiative Biology Subteam

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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.

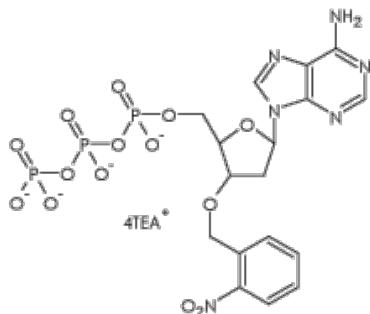


Figure 1: 3'-O-(2-nitrobenzyl)-2'-dATP

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 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
- 100mM dNTP Stock
- 100mM dATP Stock
- 10mM ddATP Stock
- 5X Terminal Deoxynucleotidyl Transferase Buffer
- Terminal Deoxynucleotidyl Transferase Stock (15U/ 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

5.2 Attempted blocking

4. Label three PCR Tubes A, B and D, respectively
5. Pipette 9.5 μ L of nuclease free water into all three PCR Tubes
6. Pipette 4.0 μ L 5X Terminal Deoxynucleotidyl Transferase reaction buffer into all three 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 directly before use
- (a) Label a PCR Tube "**BdATP Dilute**" (make this *very* clear)
 - (b) Pipette 2.25 μ L of nuclease free water into PCR Tube
 - (c) Pipette 0.25 μ L of 3'-O-(2-nitrobenzyl)-2'-dATP stock into PCR Tube
 - (d) Keep in **dark** and vortex directly before use
8. Pipette 0.5 μ L of primer into all three 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. Pipette 2 μ L of ddATP **10mM stock** into PCR Tube D
 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 [3].

13. Label two PCR Tubes C and X, respectively
14. Pipette 9.5 μ L nuclease free water into PCR Tube C (see above, **ATTEMPTED BLOCKING**)
15. Pipette 9.5 μ L of nuclease free water into PCR Tube X (see above, **CONTROLS**)
16. Pipette 0.5 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 (15 U/ μ L) into **PCR Tube C** (note, this is not the same as the last procedures. There was an error when the Terminal Deoxynucleotidyl Transferase was procured that resulted in the use of the 15U/ μ L stock rather than the 20U/ μ L stock.)
22. Incubate **all** samples at 37°C for 30 minutes

RECOMMENDED STOP POINT



5.4 Analysis

5.5 XCell Surelock Setup and Pre-Run

23. Remove 20% polyacrylamide gel from pouch and rinse with deionized water.
24. Peel off tape on bottom of 20% polyacrylamide gel and remove the comb.
25. Gently wash every cassette well with 1X TBE buffer. Invert to remove buffer and shake. Repeat twice.
26. 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.
27. 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.
28. 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.
29. 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.
30. Fill the Upper Buffer Chamber (between the gels) with running buffer. Ensure it is not leaking.
31. Fill the Lower Buffer Chamber completely with running buffer by pouring TBE next to the Gel Tension Wedge.
32. Pipette 12 μ L of running buffer into each gel well.
33. 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.

5.5.1 Run Gel

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

Figure 2: Wells

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

34. Obtain a sizable piece of parafilm. Pipette 5 μ L of Gel Loading Buffer in a row of 15 droplets.
35. For the 10/60 Ladder samples, pipette 1uL of 10/60 Ladder and 4 uL of running buffer and mix.
36. For the 'blank' droplets, pipette 5uL of running buffer.



37. For the remaining droplets, add 3.5 μL of the appropriate sample along with 1.5 μL running buffer. See the corresponding table for sample location and order.
38. As you go, pipette up and down to mix thoroughly.
39. Load the gels (with 10 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.
40. If running a second gel, repeat steps 15-18, then pipette only 5 μL of each sample into each well.
41. Run the gel(s) at 150V until the dark blue dye is at the bottom. If the dark blue dye is not visible, run the gel for three hours.

5.5.2 Stain & View Gel

42. While the gel runs, prepare 1X SYBR Gold Staining Solution with TBE as dilute
43. Once gel has finished running, *lightly* agitate gel while submerged in solution for 40 minutes.
44. Review gel with gel viewer. Until unnecessary, place gel back in stain for 20 minute increments and re-image.
45. 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 Result Analysis

6.1 Summary

While our analytic techniques provided exemplary data, the data we did collect is in contradiction with our past results from the original 3'-O-(2-nitrobenzyl)-2'-dATP Incorporation Detection Experiment. Taken in isolation, this experiment suggests that the 3'-O-(2-nitrobenzyl)-2'-dATP *does not* hinder extension (eg. does not block further extension or cannot be incorporated).

6.2 Gel Analysis

In the gel shown in Figure 6.2, we have a variety of samples: from left to right, 10/60 Ladder, Custom Ladder, B (Blocked Sample), X (Unmodified Control), B+X, D (Blocked ddATP Control), B+X, A (dATP Extended Control), C (dNTP Extended Control), 10/60 Ladder, 26 mer, 25 mer, Custom Ladder, Custom Ladder and Blank. For easy reading, the order is also enumerated in figure 5.5.1.

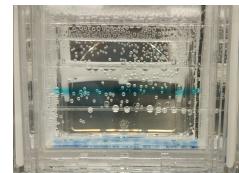


Figure 4: Gel When Stopped

Add reference here to our procedures

In direct contrast to our last two experiments, the analysis PAGE gel of this experiment provided us with useful results with accurate single base pair resolution. This is made evident by the three samples of Custom Ladder that were added to wells 2, 13 and 14. Moreover, the 26mer and 25mer were separated in adjacent wells similarly to the way the 26mer and the 25mer were separated as part of the Custom Ladder in the same well. This is important because it allows us to (1) compare multiple bands *inside the same well* and (2) compare multiple bands *across multiple wells*. This is likely due to the lack of smiling and the long run time. See Figure 5 is a picture of our gel mid run (after about 40 minutes). Throughout its run, the gel did not appear to have exceedingly different run rates for different gels. Moreover, the gel was run for a total of three hours and 10 minutes. It was stopped once the loading dye reached the bottom of the gel, see Figure 4 for future reference.



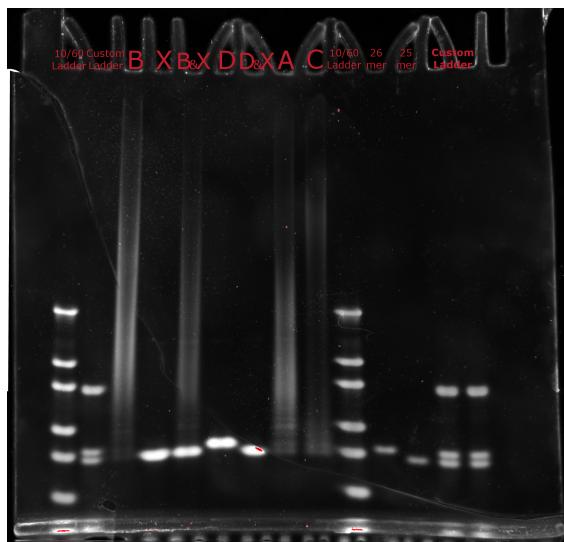


Figure 3: A digitally touched up 20% SDS PAGE Gel

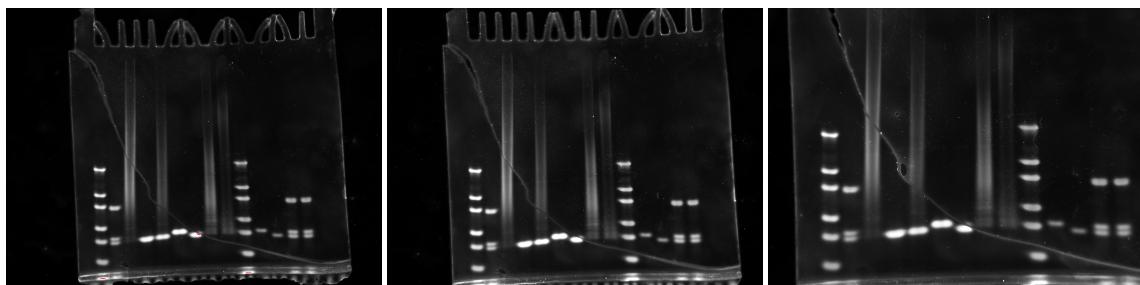


Figure 6: Unedited Gel Images

However, while the analytical techniques were not the sour-note of this experiment, the results from this experiment provide a direct contradiction of our 3'-O-(2-nitrobenzyl)-2'-dATP Incorporation Detection experiment performed on September 28, 2017 (a month before this experiment was performed). Moreover, every control sample (with the exception of D+X) exhibited either (1) the same behavior as in the original 3'-O-(2-nitrobenzyl)-2'-dATP Incorporation Detection experiment or (2) alternate behavior that we predicted would result from switching from agarose to polyacrylamide for our gel electrophoresis medium. The discrepancy in question is the extension of Sample B. In the original 3'-O-(2-nitrobenzyl)-2'-dATP Incorporation Detection experiment, we *did not* see extension of Sample B (see Figure ??). In this experiment we *did* see extension of Sample B. Moreover, there is little indication that the extension of Sample B by Terminal Deoxynucleotidyl Transferase was in any way hampered by the presence of the 3'-O-(2-nitrobenzyl)-2'-dATP or the original 3'-O-(2-nitrobenzyl)-2'-dATP extension step.

The most likely explanation of this discrepancy is that either (1) we made an error while performing the experiment and/or that (2) this is some other discrepancy between this procedure and the original procedure that produced this anomaly.

Since we have conflicting results, this or, more likely, a modified version of this procedure will need to be run.



Figure 5: Our SDS PAGE gel mid run. Notice the absence of "up-side down smiling".

Discuss this anomaly and pour over the two procedures to identify discrepancies at the biology-terminator meeting



6.3 Procedure Notes

A few mistakes were made in this procedure:

- The wrong concentration of Terminal Deoxynucleotidyl Transferase stock was used. This should have been avoided. The procedure made it clear enough, and the mistake was due solely to a lack of experience. This cause is **positive** when it comes to prevention as one will only grow more experienced, helping to ensure that the same mistake will not be made again for the same reasons.
- The 0.2 μ L-2 μ L micro pipette in the lab was not able to pipette 0.25 μ L of 3'-O-(2-nitrobenzyl)-2'-dATP for the 3'-O-(2-nitrobenzyl)-2'-dATP dilute. The pipette was set to 0.5 μ L and an estimated 0.2 μ L to 0.75 μ L of 3'-O-(2-nitrobenzyl)-2'-dATP stock was used in lieu of 0.25 μ L. This will be fixed by either (1) changing the amount of 3'-O-(2-nitrobenzyl)-2'-dATP being utilized or (2) using the new 0.1 μ L-1 μ L pipette that SSI Biology recently acquired.

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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.

