

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

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Not Peer Reviewed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.

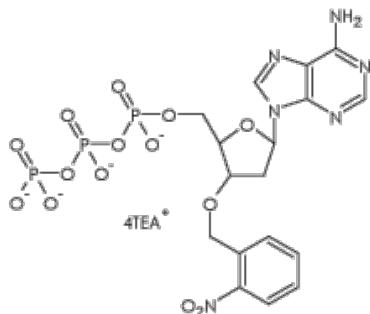


Figure 1: 3'-O-(2-nitrobenzyl)-2'-dATP

## 2 Overview

This lab will attempt to append 3'-O-(2-nitrobenzyl)-2'-dATP to a **very short (5bp) 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.[?]
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: TCATC
- 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 (20U/ $\mu$ L) **Note:** Only one stock solution is at 20U/ $\mu$ L, **be sure you have the right one.**
- Nuclease Free Water
- TBE Buffer
- 15% Urea Denaturing Gels
- SYBR Gold

**Note:** If the bioanalyzer is a valid analysis method, make a "copy" of PCR Tube B. Then store the second B as per the Stop Procedure after completion of step 13. This →  will be checked if the second B should be made.

### 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 10 $\mu$ L of nuclease free water into both PCR Tubes
6. Pipette 4.0 $\mu$ L 5X Terminal Deoxynucleotidyl Transferase reaction buffer into both PCR Tubes
7. Dilute Nucleotides:
  - (a) Label a PCR Tube "dATP Dilute"
  - (b) Pipette 9 $\mu$ L of nuclease free water into PCR Tube
  - (c) Pipette 1 $\mu$ L of dATP stock into PCR Tube
  - (d) Vortex directly before use

NOTE: This step can be skipped, as this dilute has already been made. The dilution instructions are redundant, and are here in case we want to repeat the experiment in the future.

- (a) Label a PCR Tube "BdATP Dilute" (make this *very* clear)
- (b) Pipette 9 $\mu$ L of nuclease free water into PCR Tube
- (c) Pipette 1 $\mu$ L of 3'-O-(2-nitrobenzyl)-2'-dATP stock into PCR Tube
- (d) Keep in **dark** and vortex directly before use
8. Pipette .5 $\mu$ L of primer into all three PCR Tubes
9. Pipette 2 $\mu$ L of dATP dilute into PCR Tube A
10. Pipette 2 $\mu$ L of 3'-O-(2-nitrobenzyl)-2'-dATP dilute into PCR Tube B
11. Pipette 2 $\mu$ L of ddATP **10mM stock** into PCR Tube D
12. Gently pipette 1.5 $\mu$ L Terminal Deoxynucleotidyl Transferase (**20 U/ $\mu$ L**) into all three PCR Tubes
13. Incubate sample at 37°C for 30 minutes

Note: **Do NOT** deactivate Terminal Deoxynucleotidyl Transferase

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### OPTIONAL STOP POINT

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## 5.3 Extending

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

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

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### RECOMMENDED STOP POINT

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## 5.4 Analysis

### 5.4.1 Prepare Gel

24. Prepare a 15% Denaturing Urea-PAGE gel.

Note: PAGE Gels are stored in the cold room. If 15% PAGE gels are not available, use 20% denaturing urea PAGE gels as a substitute.

- (a) Do not invert the gel at any point.
- (b) Get help from Cynthia, Lillian or Alan if possible. If uncomfortable, freeze samples.
- (c) Remove clip from gel with special tool. If tool is not available, be very careful.
- (d) After the gel and its associated apparatus and buffers have been situated, rinse each well with 15 uL of .5X TBE buffer.

25. Allow to pre-run at 200V for 20 minutes prior to continuing.

### 5.4.2 Run Gel

NOTE: Ensure that the loading dye has Bromophenol Blue in it.

26. Add 8μLof samples A, B, D, C and X with 1.5μL of loading dye for a total volume of 8μLto gel respectively, left to right with the wells at the top.
27. Add 10μL of Loading Dye to the well twice to the right of X (the seventh well from the left).
28. Add an appropriate amount of ladder (annotate the amount added on this sheet) the the far right well (wells at the top).
29. Add a 50/50 combination of sample **B** and sample **C** to the sixth well. That is, add **3μL** of sample **B**, **3μL** of sample **B** and **1.2μL** of loading dye to the sixth well, from the left with the wells at the top.
30. Run at 200V
31. Remove gel once the Bromophenol Blue has almost reached the  $\frac{3}{4}$  point [?]

### 5.4.3 Stain & View Gel

32. While the gel runs, prepare 1X SYBR Gold Staining Solution with TBE as dilute
33. Once gel has finished running, *lightly* agitate gel while submerged in solution for 40 minutes.
34. Review gel with gel viewer. Until unnecessary, place gel back in stain for 20 minute increments and re-image.
35. 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

### 6.2 Gel Analysis



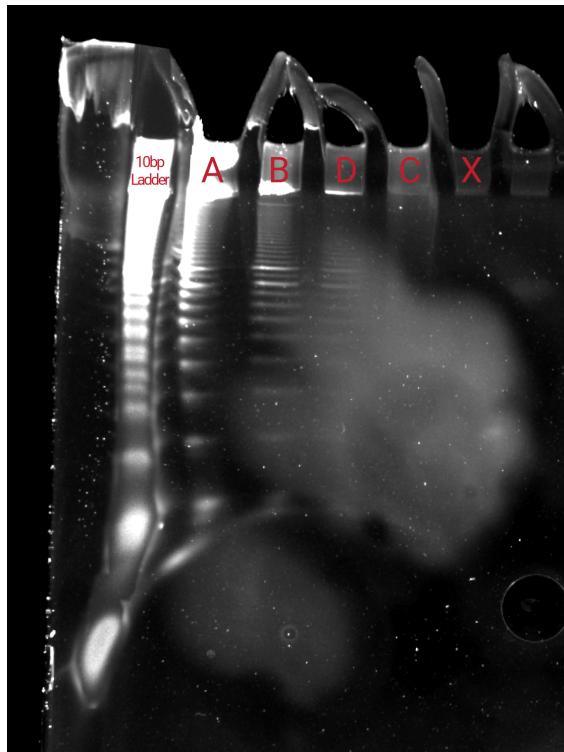


Figure 2: Digital Combination of PAGE gels from the Third Run of this experiment

### 6.3 Hybrid Gel Analysis

This "gel" is a digital combination of multiple exposure lengths. The gels were combined to increase the visibility of our "samples". The gels are pictured unedited below in Figures ?? & ???. From left to right, we *should* have a ThermoFisher 10bp Ladder, samples A, B, D, C and X. The lane to the right X had  $5\mu\text{L}$  of loading dye in it. However, judging from the results, this is **not** an accurate description of the actual contents of the gel. Before we discuss our hypothesis for what went wrong, let us address what ideally *should* have happened.

In lane 1 (numbering left to right) we should have seen a ladder resembling the ladder to the right in Figure ???. Figure ?? depicts the same 10bp ladder from ThermoFisher on an identical gel. Moreover, it was run via the same protocol as our "Perfect Page III" that this procedure's gel based analysis section was modeled after. The main differences between this procedure and that of Perfect Page III (other than the DNA being analyzed) is the amount of ladder added. In this procedure, we added  $1\mu\text{L}$  of stock while in Perfect Page III only  $0.5\mu\text{L}$  was added. This was done because the  $0.5\mu\text{L}$  added in the initial agarose based 3'-O-(2-nitrobenzyl)-2'-dATP Incorporation Detection resulted in a ladder that was too weak after 40 minutes of staining. Our concentrations of DNA were between the second and third dilutions of Perfect Page III, both of which appeared satisfactorily. As such, *given that there was no human error* the result above should not have resulted.

In row A, we should have seen a smear characteristic of a standard Terminal Deoxynucleotidyl Transferase extension. Whether or not this smear is present isn't clear from the images collected.

In row B, we should have seen a 6bp oligo due to the addition of a 3'-O-(2-nitrobenzyl)-2'-dATP to our initial 5bp primer. These predicted results are not visually present in the images we collected. Nor, however, is the smear characteristic of a standard Terminal Deoxynucleotidyl Transferase extension that would have appeared had there been an issue with the performance of the 3'-O-(2-nitrobenzyl)-2'-dATP.

In row D we should have 6bp oligo due to the addition of a dideoxyadenosine triphosphate to our initial 5bp primer (similar to row C). These predicted results are not visually present in the images we collected. Nor, however, is the smear characteristic of a standard Terminal Deoxynucleotidyl Transferase extension that would have appeared had

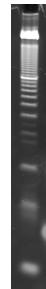


Figure 3: Ideal Ladder

there been an issue with the performance of the dideoxyadenosine triphosphate.

In row C, we should have seen a smear characteristic of a standard Terminal Deoxynucleotidyl Transferase extension. Whether or not this smear is present isn't immediately clear from the images we collected, but there does seem to be some smearing (see Figure ??).

In row X, we should have seen a 5bp oligo. This was the only reagent present in the sample. Again, this is not visually present in the images we collected.

We also should have seen an even travel of the samples. We did not. What we did see was a "upside down smile" like depicted in Figure ???. This is likely due to (1) too much current (and by relation, too much voltage) (which was never noticed in Perfect Page I, Perfect Page II, or Perfect Page III) or (2) the gel was not pressed securely against the gaskets which allowed buffer to leak out[?]. It seems that the gel was not pressed securely against the gaskets, as we did not see similar behavior in Perfect Page I, Perfect Page II, or Perfect Page III.

The question remains: why is there ladder in the rows, A, B and D? And why does the intensity of the ladder decrease as the rows get farther and farther away from the ladder row? I propose a two fold explanation: First, the ladder was prepared at too high of a concentration. This prediction rests on the fact that the ladder is much *much* brighter when imaged than we expected. Secondly, when the ladder was pipetted into row 1, a small amount of ladder was present on the *outside* of the pipette tip. When the tip entered the buffer, it fell from the tip into wells A, B and D. Moreover, the motion of the tip from left to right would have created a fluid motion conducive to more DNA ending up in the well of A rather than the well of D. Given that the ladder was sufficiently over concentrated, a single  $\mu\text{L}$  present on the tip would be more than enough to result in a gel like Figure ???. This could have been prevented by (1) preparing the ladder correctly and (2) being *extra* careful when pipetting samples into the SDS-PAGE gel. This is the most convincing hypothesis of error yet. It also implied that our sample is in the gel, we just can't see it. It also means that *no scientifically sound conclusions* regarding 3'-O-(2-nitrobenzyl)-2'-dATP and its efficiency of incorporation can be made from the images collected in this experiment.



Figure 4: Upside-down Smile on SDS-PAGE Gel(via ResearchGate)

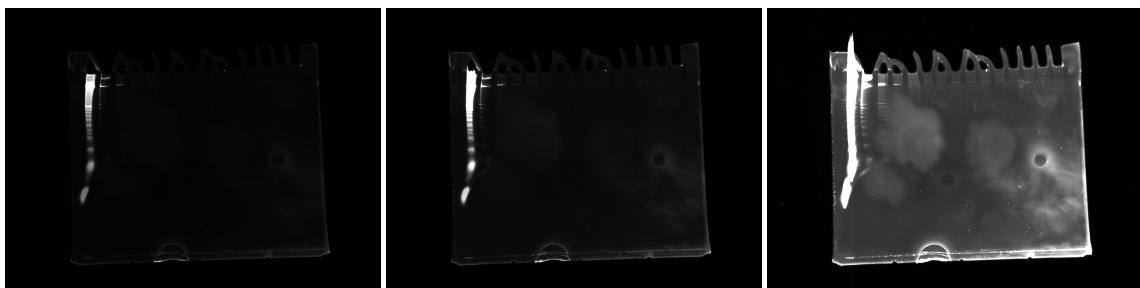


Figure 5: Unedited Gels from Version 3

From left to right, we have the respective exposure times: 0.1 seconds, faint-band-optimized and 5 seconds.

## 6.4 Procedure Notes

None not already addressed. In summary, we should be more careful when pipetting samples into gels and we need to ensure that the buffer levels on our SDS-PAGE gels is sufficiently high.

## List of Figures

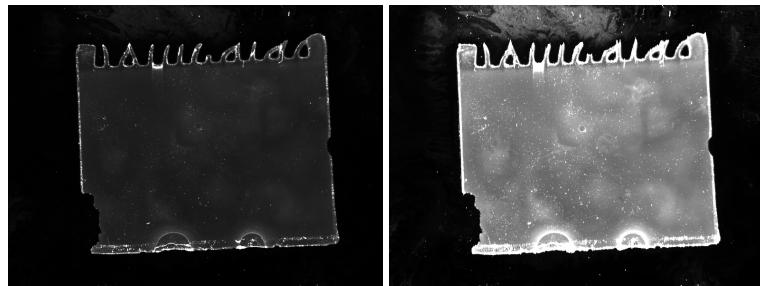


Figure 6: Unedited Gels from Version 2

Included for sake of completeness. This procedure did not succeed, likely because we did not remove the tape from our SDS-PAGE gel. Both gels have been stained for 40min, with the one on the left optimized for faint-bands and the one on the right overexposed for 10 seconds.