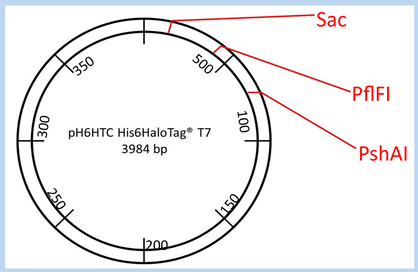
Fluorescent visualization of *in vitro* mitochondrial DNA transcription

Kelsey Bruce, Megan Bestwick

Linfield College Chemistry Department, McMinnville, OR

Introduction

Mitochondria are important organelles within eukaryotic cells especially for their role in metabolism and ATP production by the oxidative phosphorylation (OXPHOS) pathway[1]. In human cells there are approximately 80 protein subunits that make up the OXPHOS pathway; thirteen of which are encoded by the mitochondrial genome (mtDNA)[2]. Mitochondria house all the transcription and translation machinery (i.e. mitochondrial RNA polymerase, mitochondrial ribosome, tRNAs, etc.) required to produce those thirteen mtDNA encoded subunits. *In vitro* mitochondrial transcription is a method that utilizes recombinantly purified proteins and linear mitochondrial DNA templates to investigate transcription regulation of the organelle. To visualize the products of in vitro transcription, it is still common practice to utilize radioactive nucleotides or staining with ethidium bromide. These conditions can be undesirable due to safety hazards, expense, interference with electrophoresis, and time demands. As an alternative, fluorescent dyes have been developed for DNA and RNA tagging.



Model 1: the pH6HTC His6HaloTag® T7 plasmid has a total length of 3984 bp. SacI cuts at 123 bp, PflFI cuts at 418 bp, and PshAI at 715 bp. When combining SacI and PflFI, a segment of 295 bp in length. SacI and PshAI, when used together, create a segment of 592 bp. These segments were used for *in vitro* transcription.

Methods

- pH6HTC His6HaloTag® T7 DNA was transformed into competent DH5α cells for cloning
- DNA was double digested with SacI HF and PflFI or PshAI enzymes and separated on 1% low melting point agarose via gel electrophoresis in TBE buffer.
- DNA was recovered from the gel using the freeze and squeeze recovery method.
- *in vitro* transcription was completed with T7 polymerase
  - Total reaction volumes of 200 µL were used
  - Approximately 400 ng of DNA template was included in each reaction
- Products were run on PAGE under varying conditions
  - Initially, 5% acrylamide was used for casting the full gel
  - 2.5% acrylamide buffer layer was added to the top of the 5% gel
  - Electrophoresis occurred at 100-300 V for 1.5 - 12 hours
- Post staining occurred under various conditions
  - Staining with a 1% gel red dye occurred overnight
  - Staining with 1% SYBR Gold dye for 10 minutes
  - Images were taken with a BioRad EZ Imager and auto focused.

Results

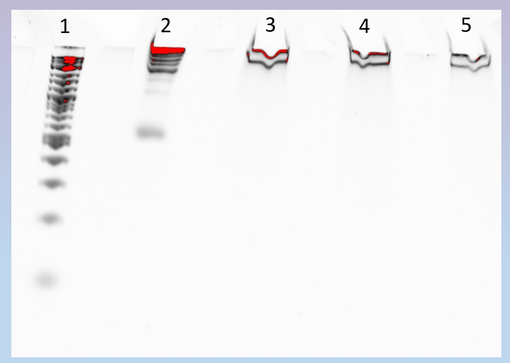


Figure 1: RNA on 5% PAGE run for 3 hours at 150 V, post stained with 1% gel red over night. Lane 1 contains 5 µL NEB 2-log DNA ladder. Lane 2 contains 3 µL 0.28-6.6 kb RNA markers . Lane 3 contains 400 ng DNA template and 2 µL T3 polymerase. Lane 4 contains 400 ng DNA template and 4 µL T3 polymerase. Lane 5 contains 200 ng DNA template and 2 µL T3 polymerase.

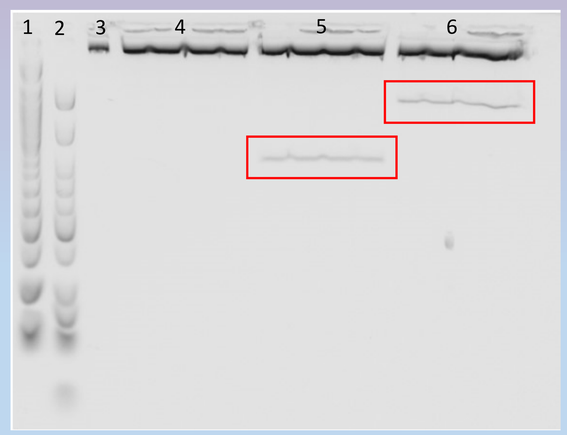


Figure 2: Double digested plasmid DNA on 2% low melting point agarose, electrophoresed at 100 V for 1 hour 23 minutes, then 20 V for 28 minutes. Lane 1 contains 5 µL 50 bp ladder. Lane 2 contains 5 µL low molecular weight ladder. Lane 3 contains DNA digested with SacI only. Lane 4 contains DNA digested with SacI and PflMI. Lane 5 contains DNA digested with SacI and PflFI. Lane 6 contains DNA digested with SacI and PshAI. Highlighted regions show where DNA was recovered for use in transcription.

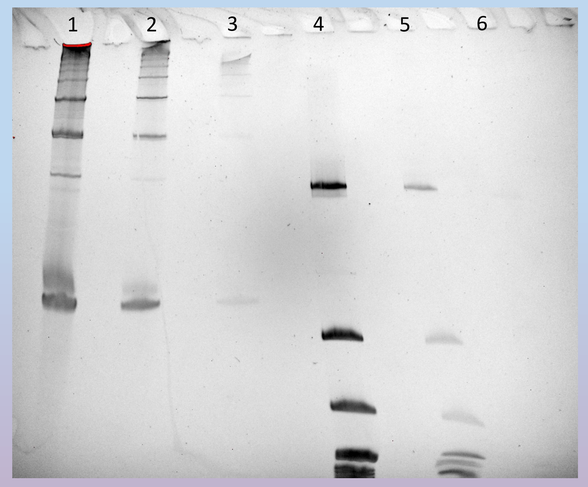


Figure 3: DNA and RNA on 5% PAGE run at 300 V for 3 hours, post stained with 1% SYBR Gold for 10 minutes. Lane 1 contains 3 µL 0.28-6.6 kb RNA markers. Lane 2 contains 0.3 µL RNA markers. Lane 3 contains 0.03 µL RNA markers. Lane 4 contains 1 µL low molecular weight DNA ladder. Lane 5 contains 0.1 µL DNA ladder. Lane 6 contains 0.01 µL DNA ladder.

Results

- Double digested DNA segments produce transcripts small enough to enter the 5% PAGE gel
- The freeze and squeeze method for recovering DNA from low melting point agarose gel provided higher yields than kits from Qiagen or Zymo.
- SYBR Gold successfully stains single strand RNA in less time than gel red.
- Transcripts had difficulty entering PAGE gels without a 2.5% buffer region
- PAGE buffer regions of less than 2.5% lack sufficient acrylamide to polymerize efficiently and solidify

Conclusions and Future Directions

SYBR Gold is an effecting dye for visualizing the single strand RNA products of in vitro transcription. A buffer region of low density PAGE gel is needed for RNA to enter the gel but current products are of a small size (<600 bp). Investigation into longer template lengths is needed to determine the appropriate use of this technique. Current work has been exclusively with plasmid DNA, but the goal is to use the same or a similar assay to study mitochondrial DNA transcription. This requires using mtDNA to test the assay, which is much more delicate and costly to procure. If successful, this assay will be used to study how protein levels regulate mtDNA transcription and expression.

References

1. Bestwick, Megan L and Gerald S Shadel. "Accessorizing the human mitochondrial transcription machinery" *Trends in biochemical sciences* vol. 38,6 (2013): 283-91.

2. Bonawitz, Nicholas D., et al. "Initiation and Beyond: Multiple Functions of the Human Mitochondrial Transcription Machinery." *Molecular Cell*, vol. 24, no. 6, 28 Dec. 2006, pp. 813–825., doi:10.1016/j.molcel.2006.11.024.

Acknowledgements

- Linfield College Chemistry Department
- Murdock Charitable Trust
- Linfield Student Faculty Collaborative Research Grant
- National Science Foundation
- Wendell C. Foote Fund