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Illustrated Protocols to Improve Undergraduate Student Research Independence

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One big challenge for undergraduate research students is gaining independence in the laboratory. In this curricular project, undergraduate students transformed research protocols developed for experienced scientists into protocols understandable to someone new to a laboratory. This process enabled themselves and other students to more quickly learn and master new techniques and advance to independent projects. Typically, students started with an original research protocol that assumed basic knowledge, such as instructions that came with a kit (i.e. plasmid purification kit instructions). Students created notes that explained the purpose of each step and reagent and provided example calculations. Then students illustrated the protocols with photos of materials needed, equipment used, action shots of difficult steps and screenshots of software programs. This approach has been used by students in laboratory courses and by new independent research students learning laboratory techniques. In the laboratory courses where students contributed to this project as part of a writing assignment, additional professional experience was gained by presenting a talk about their completed Illustrated Protocols to their classmates and by creating group posters that were presented at an undergraduate research symposium. After completion of this activity, undergraduate students gained confidence by applying their new knowledge to create user-friendly protocols. Students reported increased understanding of what is happening in each step, while instructors reported increased student independence and confidence that the protocol was being applied correctly and consistently. Thus, designing Illustrated Protocols enhanced learning and independence for the students creating the protocol and provided valuable help for future students.

Keywords: laboratory preparation, molecular biology, teaching assistant, zebrafish

Introduction

Undergraduate students are often intimidated and frustrated when learning new procedures in the research laboratory. These difficulties come from the fact that most protocols are written for experienced scientists and assume basic knowledge of equipment, techniques, and vocabulary. This confusion is illustrated by the challenges students face when using common kits, such as the QIAquick® PCR Purification Kit. After reading the first two steps in the instruction insert from this kit, students

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frequently have questions. “What does ‘5 volumes’ mean?” “Where do I find Buffer PB?” “Is this mixture orange or yellow?”

Students regularly have to perform a protocol side-by-side with an experienced person several times before they are ready to carry out a protocol on their own. Even after being instructed to take notes while performing the protocol, students often cannot anticipate the information that should be added, such as where equipment is located and where reagents are stored.

Based on these experiences of our undergraduate students, we thought that it would be useful to create a style of protocol that new researchers could more readily interpret. Our solution was to have the students themselves design “Illustrated Protocols” that clarify common points of confusion that they have identified throughout their process of learning the new protocol.

First, each student (or small group of students) gains experience with a specific protocol or technique (Figure 1). Next, the original protocol is enriched through adding photos of reagents, photos of equipment, photos of how to perform difficult steps, and screenshots from software programs. Finally, the original protocol is annotated with notes about the purpose of each step, where to find reagents, where to find equipment and example calculations. The process of creating and using these protocols should help students increase their confidence and autonomy in the undergraduate research laboratory setting.

When we queried other instructors and attendees of the ViABLE 2021 workshop about challenges they had encountered with undergraduate research, we gathered a variety of responses. Many of these challenges would be either completely or partially addressed by using the Illustrated Protocols approach. For instance, student difficulties with making dilutions and converting units was a common challenge. The inclusion of template and example calculations (Appendix A) in Illustrated Protocols challenges students to do their own calculations but provides the safety nets of template and example calculations. Another common issue was that undergraduate students have trouble understanding the overall work flow. For instance, they often lack the experience to know which steps of a protocol can be pausing points, or points where it is possible to stop the protocol to finish at a later time or date. Notes on places where work flow can be flexible and where it has to be precise would fit well within the annotations of an Illustrated Protocol.

These discussions with colleagues and ViABLE attendees also elicited several ideas for expanding this Illustrated Protocol project. One important observation was that students are often unclear about what information should be recorded in a laboratory notebook. Illustrated Protocols could be coupled with guided laboratory notebook pages with prompts where students fill in critical information about their experiment. These laboratory notebook pages could include other useful tools, such as places for students to write down how each sample is labeled or a check list that students can use to indicate when a step is completed.

Other ideas would add depth and verification steps to the Illustrated Protocol project. For instance, having students begin a new Illustrated Protocol by making work-flow charts would ensure that the student has a complete understanding and also provide an excellent overview for future students. Including additional pieces, such as quizzes that test understanding, would help translate this project into larger courses.

This Illustrated Protocol project builds on many techniques known to enhance learning, such as Instructional Scaffolding (Belland 2017), as students creating the Illustrated Protocols first learn under the guidance of an experienced scientist and then repeat the protocol with support until they are independent. Further, there is evidence that the act of preparing to teach a subject to peers enhances learning (Nestojko et al. 2014). Finally, the growth of journals such as the Journal of Visualized Experiments (JoVE), which includes videos of people carrying out protocols, to include both research and educational protocols (Vardell 2015), speaks to the demand for and success of such an approach. However, data on the effectiveness of these techniques, especially at the undergraduate level, is still sparse. Thus, an important next step in this process will be to add pre and post assessment surveys which will enable us to assess whether this project has a positive effect on student learning.

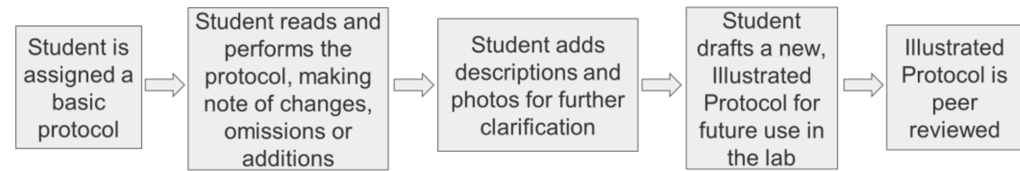

Figure 1. Workflow for the Illustrated Protocols laboratory course or research laboratory activity.

Instructions for Students

Illustrated Protocols Project

Objective

Use your experience learning a new protocol to create a new version of the protocol that includes illustrations, explanations, and notes that will enable future students to learn and master the protocol more quickly and to understand the techniques more fully.

Introduction

Gaining the confidence to perform laboratory research protocols independently can be intimidating and frustrating. Even experienced scientists face hurdles when learning a new technique. These difficulties arise because most protocols assume background knowledge and experiences. If you do not have the assumed background, it can be difficult to understand such things as why each step is performed, where to find and how to use the required equipment, and how to perform difficult steps. We have found that adding images and annotations to the research protocol can decrease these challenges by filling in this background knowledge. To help you and future students accurately and confidently perform research protocols on your own, you will be creating your own Illustrated Protocol. Be sure to keep a record of all of the work you do towards making your Illustrated Protocol, including notes with dates and clearly labeled images. Making these protocols is research, just like doing an experiment. Your Illustrated Protocol may be published, presented to others students or to a larger group of scientists at a conference. Thus, your contributions should be accurately and fully documented.

Methods

Part A: Practicing Your Protocol

The first part of this project involves performing your protocol with the assistance of someone with experience. As you observe and practice each step, start making a list of the notes and taking the photos you want to include for your Illustrated Protocol. Decide on which sections of the Illustrated Protocol (Table 1) to include each of your notes and images.

Part B: Creating Your Illustrated Protocol

To create your protocol, follow the "Template for an Illustrated Protocol" (Appendix A). Used the images you have taken to make figures to guide future students through the steps. These images can include pictures of reagents, equipment, locations, action shots of difficult steps, screenshots of software programs and anything else you find helpful. In addition, use the notes you took to annotate the protocol with information such as the purpose of key steps, where reagents and equipment can be found, how to make needed solutions, and how to do required calculations.

Table 1. Illustrated Protocol Sections

Section of Protocol	Purpose
Title	A brief statement of the scientific technique that will be performed in the protocol
Author(s)	Gives credit to you as the student developer of the protocol (and your peers that help)
Introduction	Orients students to the overall purpose and flow of the procedure
Reagents/Equipment	A quick way to see what materials and tools are needed to prepare for the experiment
Methods	Descriptive steps with images and added notes that give students clear direction on how to perform the experiment independently
Source Citations	Gives credit to the author(s) of the original protocol (often the manufacturer of the kit)

Part C: Test and Revise Your Illustrated Protocol

After you have completed your Illustrated Protocol, ask other classmates or lab-mates to use it and share their notes and ideas for making improvements and revisions. Once you have collected feedback from several users, use your own experience and judgement to make a revised version.

Examples

A **Original Protocol:**

Qiagen. 2020. *QIAquick Spin Handbook*

4. To wash, add 750 ul Buffer PE to the QIAquick column.

Illustrated Protocol adds:

4. Add 750 µl of Buffer PE (Figure 14).
   a. Buffer PE washes out unbound molecules.
   b. Plasmid DNA location is in the matrix of the QIAquick spin column.

Figure 14: Buffer PE

B **Original Protocol:**

Life Technologies. 2012. *TaqMan® RNA-to-CT™ 1-Step Kit*

1. Prepare the master mix by combining the Taqman Gene Expression Master Mix (TGEMM) and the nuclease-free H_2O (NF-H_2O).

Illustrated Protocol adds:

Equations:
10 µl TGEMM x (# of wells + ~3) = µl total volume of TGEMM
7 µl NF-H_2O x (# of wells + ~3) = µl total volume of NF-H_2O
Example for 27 wells:
10 µl TGEMM x (30) = 300 µl of TGEMM
7 µl NF-H_2O x (30) = 210 µl of NF-H_2O

Figure 2. Comparisons of original kit instructions to our Illustrated Protocols.
A) On the left is a step from the QIAquick® Spin Handbook (Qiagen 2020) and on the right is the comparable step from the matching Illustrated Protocol (Appendix C) with an added reagent image and explanations.
B) On the left is a step from the Taqman® RNA to CT^{TM} 1-Step Kit User Guide and on the right are the additional equations and example calculations in the Illustrated Protocol (Appendix D).

A

Procedure

1. Add 5 volumes of Buffer PB to 1 volume of the PCR sample, and then mix. It is not necessary to remove mineral oil or kerosene.
   For example, add 500 µl of Buffer PB to 100 µl PCR sample (not including oil).
2. If pH Indicator I has been added to Buffer PB, check that the mixture's color is yellow.
   If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0,
3. Place a QIAquick spin column in a provided 2 ml collection tube.
4. To bind DNA, apply the sample to the QIAquick column and centrifuge for 30–60 s.
5. Discard flow-through. Place the QIAquick column back into the same tube.
   Collection tubes are reused to reduce plastic waste.
6. To wash, add 0.75 ml Buffer PE to the QIAquick column and centrifuge for 30–60 s.
7. Discard flow-through and place the QIAquick column back into the same tube. Centrifuge the column for an additional 1 min.
   IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.
8. Place QIAquick column in a clean 1.5 ml microcentrifuge tube.
9. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. Alternatively, for increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge.

B

Section III. Purifying Linearized Plasmid DNA
Now we want to purify our linearized plasmid from the reaction mix. We will be using the Qiaquick® PCR Purification Kit (Figure 4). Because this kit purifies DNA segments up to 10kb in size, it will work for most linearized plasmids.

Figure 4. QIAquick® kit

5. Add 5 volumes Buffer PB to 1 volume restriction digest reaction and invert (Figure 5).

   Example:
   If you have 200 µl of restriction digest reaction, you will add 5 x 200 µl = 1000 µl of Buffer PB.

   *Note: Buffer PB helps bind the plasmid DNA to the QIAquick® column.

Figure 5. Buffer PB (left) gets added to the reaction (middle) and the solution should turn yellow (right)

6. Add pH indicator in a 1:250 ratio.

   Example:
   If you have 1200 µl of restriction digest reaction + Buffer PB you will add 1200/250 = ~5 µl pH indicator.

   *Note: The solution will turn yellow in color (Figure 5, right). This indicates that the pH is $\leq$7.5 which is needed in order for the DNA to absorb to the membrane. If the color of the mixture is orange or violet, add 10 µl 3M sodium acetate, pH 5.0, and mix until the color of the mixture turns yellow.

Figure 3. Comparison of original kit instructions to an Illustrated Protocol.
A) The original instructions from the Qiagen QIAquick® Spin Handbook (Qiagen 2020).
B) Our Linearizing Plasmid DNA Illustrated Protocol (Appendix C) where this kit is used demonstrates the addition of short introductory explanations of protocol sections, images of kit box + contents, example calculations, and images of a key color-changing step.

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Notes for the Instructor

Examples of Illustrated Protocols can be useful for students to view as they are working on this project and for instructors thinking about using this approach. Figure 2 demonstrates how images and example calculations can increase the clarity of specific protocol steps, while Figure 3 provides an extended before and after example. Additionally, we have three full-length Illustrated Protocols available for your use in Appendices B, C and D. Our protocols focus primarily on research techniques utilized for molecular biology and/or zebrafish experiments (Table 2).

Table 2. Current Illustrated Protocols

Current Illustrated Protocols
Plasmid DNA Purification (Appendix B)
Linearizing Plasmid DNA (Appendix C)
Agarose Gel Electrophoresis
Designing sgRNA Primers for CRISPR
PCR for sgRNA Primer Production
Zebrafish Embryo Injection
Identifying Carriers of Mutations and Transgenes
Brightfield Imaging on a Compound Microscope
Wholemount In Situ Hybridization
RNA Isolation
cDNA Synthesis
Taqman® qPCR (starting with cDNA)
Taqman® 1-Step qPCR (starting with RNA) (Appendix D)

We have implemented Illustrated Protocols both in the classroom and research laboratory settings. In the classroom, students in a small course-based undergraduate research experience (CURE) course (Dolan 2016) completed an Illustrated Protocol as a semester-long project that included an oral presentation of the final product. In the research laboratory, Illustrated Protocols have been used as working documents: former students designed them and current students update them as they implement the techniques. When students have insufficient time in the research laboratory to complete an original project, Illustrated Protocols have served as an excellent product demonstrating student efforts and experience with research protocols and research literature.

Feedback from students has indicated their recognition of the value of this activity. A former student that designed an Illustrated Protocol as a course assignment commented, "I think a key part of making these protocols is so that newer undergraduates can apply general chemistry and biology knowledge in a practical fashion without having to take (or remember everything from) an advanced molecular biology course." While one of our research students reflected, "I feel like [this process] made me understand the procedure more as a whole, as I wasn't able to just copy what the original said. I also think those other protocols written by previous students were way easier to understand than the ones certain kits/experiments came with."

During our ViABLE workshop discussion, participants had several valuable suggestions for how to adjust the activity for various settings. For introductory laboratory courses, a previously completed Illustrated Protocol could be provided as part of a pre-lab assignment, to help students visualize what they will be doing in class. Alternatively, students could begin with a traditional protocol or a partially completed Illustrated Protocol that they perform during lab, then have to edit as an assignment. There were many ideas on how Illustrated Protocols could be made even more useful. For instance, to make the Illustrated Protocols lab-friendly and reusable, checkboxes could be added for each step, the physical protocol could be laminated, and a dry erase marker could be used to check each step as it is completed along the way. Another possibility would be to add a lab notebook component (spaces in the Illustrated Protocol with specific prompts for information that should be recorded), so students are taking correct notes while performing the procedure. For an in-class activity or formative assessment, students could be provided with an Illustrated Protocol that has some missing information, that students then have to fill in with detail.

There are also many ways Illustrated Protocols could be adapted for more advanced students. For courses that involve learning to read and analyze primary literature or for research that uses a new published technique, students could begin with the methods section from a published study and add the images and annotations to convert the methods text into an Illustrated Protocol.

This idea could also be adapted to group work. For larger, multi-section laboratory courses, each protocol could be assigned to several sections. Then the students from each section that are working on the same protocol could peer review each other's work. This has the added benefit of giving students practice with important skill of peer review. A potential incentive would be to include the best version in the official course laboratory manual. Additionally, one protocol could be subdivided, with each course section focusing on a different part. Or for smaller courses, one protocol could be divided between several group members.

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Several uses suggested by workshop participants focused on undergraduate students involved in preparing materials for laboratory courses. Having these students make Illustrated Protocols could enrich their experience and help smooth transitions as students move in an out of these positions. In some cases, students in these assistant positions are required to go beyond just following instructions, and making an Illustrated Protocol would be a good fit for this requirement. All participating in the workshop discussions recognized many other potential uses in having Illustrated Protocols for the preparatory work. For instance, Illustrated "Prep" Protocols could be shared with students taking the course to demonstrate how much effort goes into each experiment before the lab even starts.

Workshop participants also indicated that designing Illustrated Protocols would be a useful activity at all instructional levels from high school through undergraduate education and even into graduate programs. As we continue to utilize Illustrated Protocols, we have started formally assessing their effect on student confidence and independence. We look forward to sharing the results of our assessment analysis in the near future.

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About the Authors

Hannah Campbell was an undergraduate student at the University of Minnesota Duluth, a Laboratory Technician at The College of St. Scholastica and is currently a Medical Laboratory Technician at Grand Itasca Clinic & Hospital in Grand Rapids, MN. Ryan Steger was an undergraduate student at The College of St. Scholastica and is currently a Research Associate at the Broad Institute. Jennifer Liang is a Professor in the Biology Department at the University of Minnesota Duluth and the Director of the University of Minnesota Integrated Biosciences Graduate Program. Jenean O'Brien is an Assistant Professor at The College of St. Scholastica, where she teaches first year biology majors, non-majors and upper division elective courses in cellular and molecular biology.

Appendix A

Template for an Illustrated Protocol

Created by Student X, Student Y and Your Name

List of Source Protocols
Example source list: Adapted from Thisse et al, 1993 and QIAprep® Spin Miniprep Kit

Introduction
This is a quick description of what the protocol is and its uses.

Reagent List
All of the reagents (supplies) you will need to perform this protocol are listed here

Equipment List
All of the equipment you will need to perform this protocol is listed here

Safety: Because every institution has slightly different rules about safety, you should include this blanket statement instead of specific safety information: “When carrying out this protocol, be sure to follow all safety and training guidelines set by your institution.”

Day 1 (if needed for the protocol)

Section I. Clear name for this section

1. Step one of the protocol - add period after each step. We will have an empty line between each step but not within each step.

2. Some steps will have an explanation for why that step is performed. Save these explanations for unique and/or critical steps.

3. Some steps will have extra notes added to them.
    - Some notes will be about something special that needs to be pointed out. Such as - this is a place where you could pause the protocol and continue at a later date. None of these notes should be in red font.
    - Each note can have its own bullet point if more than one note is needed for one step.

Section II. In the new section, continue numbering for each step, do not restart at #1.

4. Some steps will require calculations. Here, it will be helpful to provide a template for how to do the calculation AND an example calculation.

Example template calculation:
Using the template RNA concentration (ng/µl), set up an equation to calculate the volume of µL required to get 1000 ng of RNA:

$$\frac{Y\ ng}{1\ \mu L} = \frac{1000\ ng}{X\ \mu L}$$

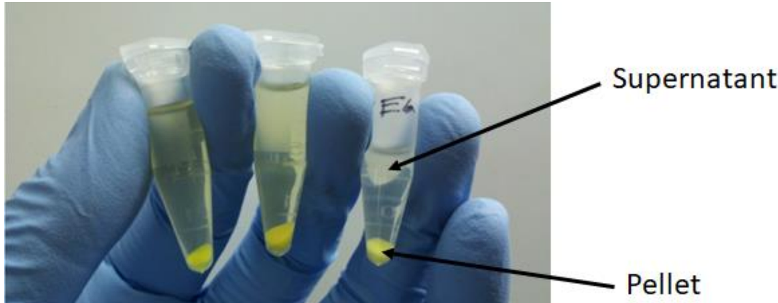
Y = concentration of RNA sample
X = volume you will use in reaction

Example calculation:

$$\frac{295.5\ \text{ng}}{1\ \mu\text{L}} = \frac{1000\ \text{ng}}{X\ \mu\text{L}} \qquad X=3.4\ \mu\text{L}$$

5. Figures and Tables should be placed underneath the step that they correspond to, as Google Docs does not format figures placed next to text very well.

Example Figure 1. This is Buffer P1, stored at room temperature. Full sentences should be used as needed. Each figure should be placed in the text where it is used, not at the beginning in the reagent/equipment lists. The figure captions should be placed underneath the figures. Image credit: Hannah Campbell



Example Figure 2. If you or someone in your team did not take the image themselves, be sure to include source information. If at all possible, use an original image. Image source[6]: https://aylo6061.com/tag/plasmid/ (Louie 2012)

Example Table 1. Each table should have a title. For example: Template for 96-well plate.

Genes of Interest	Sample 1: Non-Injected Control (NIC)			Sample 2: CDK1-Morpholino Injected (CDK)			Sample 3:			Sample 4:		
Gene 1: p53	p53 NIC	p53 NIC	p53 NIC	p53 CDK	p53 CDK	p53 CDK						
Gene 2: Ras	Ras NIC	Ras NIC	Ras NIC	Ras CDK	Ras CDK	Ras CDK						
Gene 3: 18S	18S NIC	18S NIC	18S NIC	18S CDK	18S CDK	18S CDK						
Gene 4:												
Gene 5:												
Gene 6:												
Gene 7:												
Gene 8:												

6. Here is a list of some common preferred abbreviations and symbols.
   - µl
   - °C
   - room temperature (not RT)
   - NF-H_2O is ok if spelled out (nuclease-free water) the first time it is referenced within a protocol

7. Some steps will require an indented list of reagents that get added together to a final volume, as indicated below. Be sure to list the reagents in the order that they should be added to a mixture. For example, the RT-PCR mix would be added first, then the primers/probe, then the RT enzyme mix, etc, in the list below.

   Ingredients per well:
   10 µl RT-PCR mix
   1 µl primers/probe
   0.5 µl RT enzyme mix
   ___ µl RNA
   ___ µl NF-H_2O
   20 µl total

8. The last step often indicates what is next or where to store the final product or both.

Cited References (use full citations in the Council of Science Editors name-year format)

1. Format for a journal article:
Author last name First initial. Year. Article title. Journal title. Volume(issue number):pages. doi:

2. Example from a journal article:
Thisse C, Thisse B, Schilling TF. 1993. Structure of the zebrafish snail1 gene and its expression in wild-type, spadetail and no tail mutant embryos. Development. 119:1203-1215. doi:10.1242/dev.119.4.1203

3. Format for a commercial/manufacturer's instructional guide (from kits):
Company name. Date posted/revised. Name of the protocol. Available from: URL

4. Example from a commercial/manufacturer's instructional guide:
Qiagen. 2020. QIAprep® Miniprep Handbook. Available from:
https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/plasmid-dna/qiaprep-spin-miniprep-kit/

5. Format for a blog:
Author last name first/middle initials. Blog publication date. Title of blog entry. Title of blog [Internet]. Publication City(State initials): Publisher name. [citation date]. Available from: URL.

6. Example from a blog post:
Louie A. 2012 Apr 3. GFP Project Week 3: DNA! AyLo Projects and Adventures Blog [Internet]. Boston (MA): Wordpress.com [cited 2021 Jul 15] Available from: https://aylo6061.com/tag/plasmid/

7. Format for a website:
Author last name first/middle initials. c(year published). Title of website [Internet]. Publication city(state): Publisher/Organization. [updated year abbreviated month day; cited year abbreviated month day]. Available from: URL

8. Example from a website:
APSnet: plant pathology online [Internet]. c1994-2005. St Paul (MN): American Phytopathological Association. [cited 2005 Jun 20]. Available from: http://www.apsnet.org/

Appendix B

Plasmid DNA Purification Illustrated Protocol

Created by Samantha Meek and Hannah Campbell

List of Source Protocols

Adapted from Qiagen QIAprep® Miniprep Handbook

Introduction

Plasmid purification is a process that isolates plasmid DNA from other components of bacterial cells, such as genomic DNA, cell membrane, and proteins. Purified plasmids have many uses. Plasmids can have fragments of genes or entire coding sequences of genes inserted into them. In this way, plasmids containing the protein coding sequence of a gene located after a promoter can be used to overexpress that protein. Bacteria can then be used to make many copies of these genes. As bacterial cells multiply, they make copies of the plasmid. Plasmids then need to be isolated (or purified) from the bacterial cells. The QIAprep® Spin Miniprep Kit is used for purification of up to 20 µg of DNA into a 30-50 µL volume.

Reagent List

Contents of QIAprep® Spin Miniprep kit:

- QIAprep® 2.0 spin columns
- Buffer P1
- Buffer P2
- Buffer N3
- Buffer PB
- Buffer PE
- Buffer EB
- LyseBlue
- Loading dye
- RNase A
- Collection tubes (2 ml)
- QIAprep® Spin Miniprep Kit Quick-Start protocol

Equipment List

Microcentrifuge
1.5 ml microfuge tubes
Microfuge tube rack
p1000 pipette (100-1,000 µl)
p200 pipette (20-200 µl)
p2 or p10 pipette (2-20 µl)
Pipette tips for pipettes
Liquid waste container
Pipette tip waste container
Nanodrop™ Spectrophotometer
Kimwipes™
Nuclease free water (NF-H_2O)
Nitrile gloves
Lab coat

Safety: When carrying out this protocol, be sure to follow all safety and training guidelines set by your institution.

Section I. Purifying the Plasmid DNA

1. Centrifuge the 1-5 ml overnight culture at >8,000 rpm (6800 x *g*) for 3 minutes in the microcentrifuge or centrifuge to pellet the *E. coli* bacteria.
    a. Pipette off the supernatant.
    b. Plasmid DNA location is in the pellet.

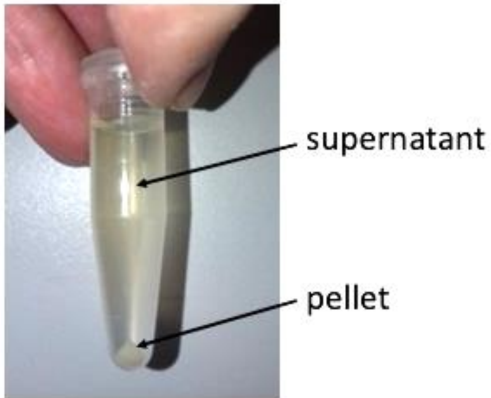


Figure 1. Example of how the microcentrifuge tubes will look after centrifugation.

2. Add 250 µL of Buffer P1 (Figure 2) and transfer to a new 1.5 mL microfuge tube (Figure 3).

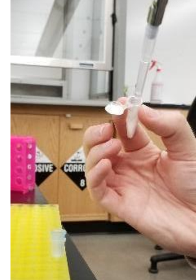


Figure 2. Buffer P1 Figure 3. Move to microcentrifuge tube

    a. Buffer P1 is used for resuspending pelleted *E. coli.*
    b. Plasmid DNA location is in the solution.

3. Add 250 µL of Buffer P2 (Figure 4) and mix by inverting the tube 4-6 times.
    a. Buffer P2 lyses the *E. coli* to release the plasmid DNA and other cellular contents.
    b. If the LyseBlue reagent was added to Buffer P2, the solution will turn blue.
    *Note: LyseBlue is optional and can be used to provide visual identification of optimal buffer mixing
    c. Plasmid DNA is in the supernatant.

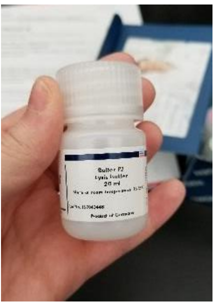


Figure 4. Buffer P2

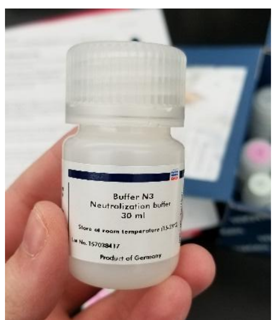


Figure 5. Buffer N3

4. Add 350 µL of Buffer N3 (Figure 5) and mix by inverting the tube 4-6 times.
    a. Buffer N3 used for neutralization of solution to renature the plasmid DNA
    b. If the LyseBlue reagent was added, the solution will now turn colorless.

5. Centrifuge for 10 minutes at 13,000 rpm (17,900 x g) (Figure 6).
    a. Plasmid DNA location is in the supernatant, the pellet contains the other cellular contents/debris.

Figure 6. Tubes loaded in centrifuge

6. Using a p1000, pipette the supernatant from step #5 into the QIAprep spin column placed inside the collection tube (Figures 7 and 8).
    a. The other cellular components (genomic DNA, cell membrane, proteins, etc.) are in the pellet from step #4 and are discarded.

b. This collection tube can be reused until the final elution of plasmid DNA.

Figure 7. Pipetting supernatant into spin column

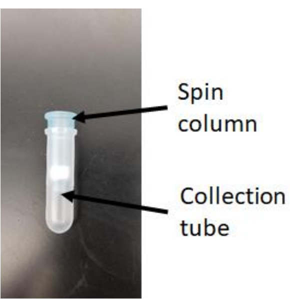


Figure 8. Spin column in collection tube

7. Centrifuge for 30-60 seconds at 13,000 rpm (17,900 x g) (Figure 9).

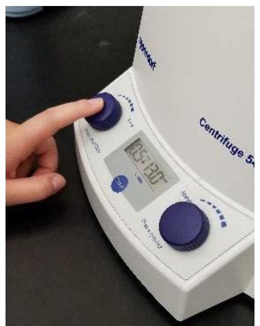


Figure 9. Set and run centrifuge

Figure 10. Flow through in bottom of collection tube

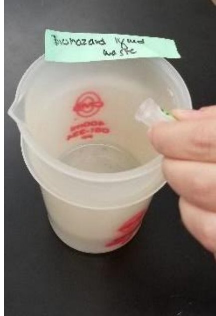


Figure 11. Discarding flow through

   a. Discard flow through from the collection tube into a liquid waste container (Figures 10 and 11).
   b. Plasmid DNA location is in the matrix of spin column.

8. Add 500 µL of Buffer PB (Figure 12).
   a. Buffer PB is used to help bind the plasmid DNA to the matrix and wash out any unbound molecules.

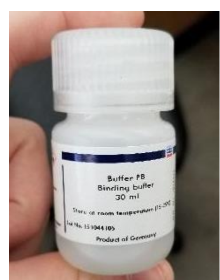


Figure 12. Buffer PB

   b. Plasmid DNA location is in the matrix of spin column.

9. Centrifuge for 30-60 seconds at 13,000 rpm (17,900 x g) (Figure 13).
   a. Discard flow through into the liquid waste container (Figure 14).
   b. Plasmid DNA location is in the matrix of spin column.

Figure 13. Set and run centrifuge

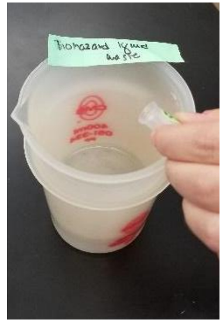


Figure 14. Discarding flow through

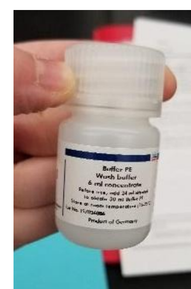


Figure 15. Buffer PE

10. Add 750 µL of Buffer PE (Figure 15).
    a. Buffer PE washes out unbound molecules.
    b. Plasmid DNA location is in the matrix of spin column.

11. Centrifuge for 30-60 seconds at 13,000 rpm (17,900 x g) (Figure 16).
    a. Discard flow through into the liquid waste container (Figure 17).
    b. Plasmid DNA location is in the matrix of spin column.

Figure 16. Set and run centrifuge

Figure 17. Discarding flow through

12. Centrifuge for 1 minute at 13,000 rpm (17,900 x g) to remove any residual wash buffers (Figure 18). Plasmid DNA location is in the matrix of spin column.

Figure 18. Set and run centrifuge

13. Transfer spin column to a new 1.5 mL microfuge tube.
    a. Label with plasmid DNA name, date, and your initials (Figure 19).
    b. Plasmid DNA location is in the matrix of spin column.

Figure 19. Properly labeled tube (left – side, right – top)

14. Using a p200 pipette, add 50 µL of EB Buffer (Figure 20) or nuclease-free water (NF-H2O) if plasmid is to be injected into zebrafish embryos to the center of the spin column.

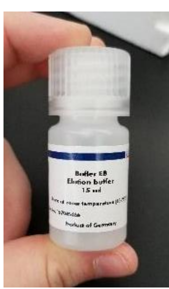


Figure 20. Buffer EB

   a. Let stand for 1 minute to allow plasmid DNA to elute into solution.
   b. EB Buffer elutes the plasmid DNA from the matrix into solution.
   c. Plasmid DNA location is now in the solution/flow through.

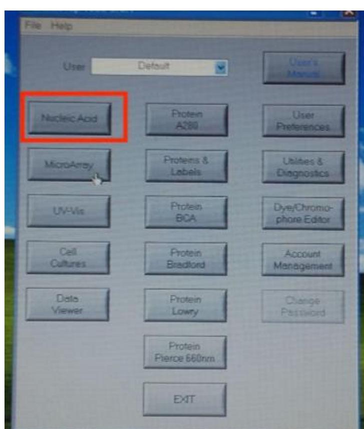
15. Centrifuge for 1 minute at 13,000 rpm (17,900 x g) (Figure 21).
Plasmid DNA location is in the flow through.

Figure 21. Set and run centrifuge

Section II. Determining the Plasmid DNA Concentration

16. Select Nucleic Acid Settings on the startup of the Nanodrop™ program on the lab computer (Figure 22).

Figure 22. “Nucleic Acid” setting is marked with a red rectangle



17. Blank Nanodrop™ with Buffer EB or NF-H_2O, whichever you eluted your plasmid DNA into, by placing 1 uL of EB (or NF-H_2O) on Nanodrop™ sensor (Figures 23 and 24). Choose “blank” within the Nanodrop™ software program.
18. Wipe away blank solution with wet Kimwipe™ (Figure 25) and place 1 µL of eluted plasmid DNA solution on the Nanodrop™ sensor using a p2 or p10 pipette.

Figure 23. Buffer EB

Figure 24. Adding blanking solution to sensor area of Nanodrop™

Figure 25. Cleaning Nanodrop™

19. Click “Measure” in the Nanodrop™ software.

20. Record the concentration and absorbance ratios (Figure 26) for the eluted plasmid DNA in your laboratory notebook.

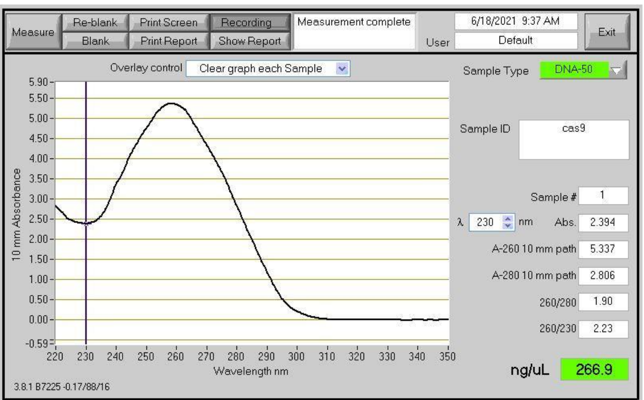


Figure 26. Screenshot showing concentration (ng/µl) and absorbance (Abs) readings

21. Clean Nanodrop™ sensor with Kimwipe that has been moistened with some NF-H_2O.

22. Calculate total yield of plasmid and record in laboratory notebook.
    Equation: concentration of DNA solution X total volume = yield
    Example calculations:
    267 ng/µl X 50 µl = 13,350 ng
    13,350 ng X 1 µg/1000 ng = 13.35 µg total yield

23. Store purified plasmid DNA at -20°C.

Cited References

1. Qiagen. 2020. QIAprep® Miniprep Handbook. Available from: https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/plasmid-dna/qiaprep-spin-miniprep-kit/

Appendix C

Linearizing Plasmid DNA Illustrated Protocol

Created by Hannah Campbell

List of Source Protocols

Adapted from J.O. Liang laboratory protocols and Qiagen QIAquick® Spin Handbook

Introduction

In this example, we are preparing plasmid DNA for use as a template for transcription of mRNA. The linearized (cut) plasmid is used as the template in an *in vitro* (in a test tube) reaction in which the mRNA is synthesized. If plasmid DNA is not linearized, it will remain circular and the RNA polymerase could continue transcribing past the gene of interest. Linearizing the plasmid will prevent the transcription of a jumble of unnecessarily long RNAs. Thus, linearization of the plasmid also increases transcription efficiency.

The plasmid contains (Figure 1), in the following order: a basal promoter sequence that enables binding of a RNA polymerase. In this case, the promoter is for the bacteriophage T3 RNA Polymerase. After the promoter, the plasmid contains the gene of interest for transcription. In this case, the gene is Cas9. The gene is typically a complementary DNA (cDNA), thus it has the start site, the protein coding sequence of the gene, the stop site, and the sequence that encodes the polyA addition at the end of the mRNA. Because it is a cDNA, the gene does not contain introns. After the gene is the recognition site for the restriction enzyme (RE) that will cut (and therefore, linearize) the plasmid, in this case Xba1.

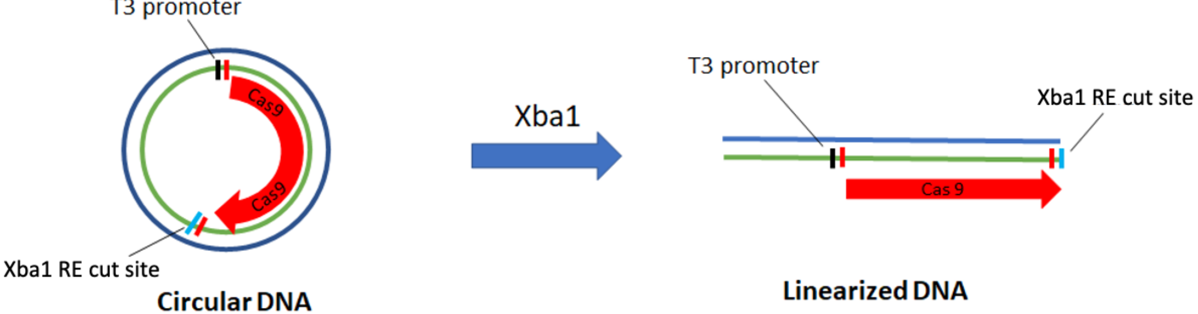


Figure 1. Using Xba1 restriction enzyme to cut circular plasmid DNA to create linearized DNA

Reagent List

Nuclease-Free water (NF-H_2O)
Plasmid DNA
BSA (comes with Restriction Enzyme)
10X Restriction Enzyme Buffer (comes with Restriction Enzyme)
Proteinase K enzyme
2X Proteinase K buffer (0.2 mM Tris, pH7.5, 25 mM EDTA, 0.3 M NaCl, 2%SDS)
Restriction Enzyme (example from above: Xba1)
Ice

Reagents included in QIAquick® PCR Purification Kit

PB Buffer
PE Buffer

pH indicator
3M Sodium Acetate
Qiaquick® columns
2 ml Collection tubes

Equipment List
Microcentrifuge
Pipettes
Pipette tips
Waste container
1.5 ml microcentrifuge tubes
Nanodrop™ machine
Kimwipes™
Ice bucket

Safety: When carrying out this protocol, be sure to follow all safety and training guidelines set by your institution.

Section I. How to calculate amount of plasmid DNA to add to reaction mixture

1. You will need to calculate how many microliters of plasmid DNA you need to add 10 micrograms of total plasmid to your mixture based on the concentration of your plasmid DNA.

Example: $10\ \mu g \times \frac{1\ ml}{350.6\ \mu g} \times \frac{1000\ \mu l}{1\ ml} = 28.5\ \mu L$

10 μg = amount of plasmid you want to add to reaction

350.6 μg/ml = concentration of your plasmid DNA determined by the Nanodrop™

28.5 μl = plasmid DNA volume needed for reaction mixture, your volume will be different based on your calculations. For example, I found that I needed to add 28.5 μl of my Cas9 Plasmid DNA.

*Note: The total volume of the reaction mixture needs to be 100 μl.
100 μl - 28.5 μl plasmid DNA - 10 μl 10X buffer - 1 μl BSA - 2 μl restriction enzyme = 58.5 μl.
Therefore, I added 58.5 μl nuclease-free water (NF-H_2O) to reach a total volume of 100 μl for the reaction mixture.

Section II. Making reaction mixture

2. Make reaction mixture by combining the reagents below.
*Note: Always add the restriction enzyme last. Keep restriction enzyme on ice at all times.

Volume	Reagent
__ μl	circular plasmid DNA (10 μg)
10 μl	10x Restriction Enzyme buffer
1 μl	BSA (Figure 2)
__ μl	NF-H_2O (Figure 2)
2 μl	Restriction Enzyme (Figure 2)
100 μl	Total volume

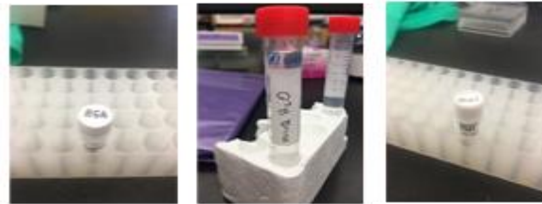


Figure 2. BSA (left), nuclease-free or milliQ water (middle) and the restriction enzyme Xba1 (right)

3. Incubate reaction mixture anywhere from 2 hours to overnight at 37°C.

4. To stop the reaction by degrading the restriction enzyme:
    a. Add 100 µl 2x Proteinase K *buffer* to the digest (Figure 3).
    b. Add 1 µl of 100-200 µg/ml Proteinase K to the digest.

Figure 3. Proteinase K buffer

Section III. Purifying Linearized Plasmid DNA
Now we want to purify our linearized plasmid from the reaction mix. We will be using the Qiaquick® PCR Purification Kit (Figure 4). Because this kit purifies DNA segments up to 10kb in size, it will work for most linearized plasmids.

Figure 4. QIAquick® kit

5. Add 5 volumes Buffer PB to 1 volume restriction digest reaction and invert (Figure 5).

Example:
If you have 200 µl of restriction digest reaction, you will add 5 x 200 µl = 1000 µl of Buffer PB.

*Note: Buffer PB helps bind the plasmid DNA to the QIAquick® column.

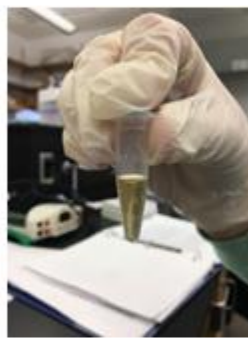


Figure 5. Buffer PB (left) gets added to the reaction (middle) and the solution should turn yellow (right)

6. Add pH indicator in a 1:250 ratio.

Example:
If you have 1200 µl of restriction digest reaction + Buffer PB you will add 1200/250 = ~5 µl pH indicator.

*Note: The solution will turn **yellow** in color (Figure 5, right). This indicates that the pH is $\leq$7.5 which is needed in order for the DNA to absorb to the membrane. If the color of the mixture is orange or violet, add 10 µl 3M sodium acetate, pH 5.0, and mix until the color of the mixture turns yellow.

Section IV. Column Prep and DNA Binding

7. Place a QIAquick® column into a 2 ml collection tube (Figure 6).

Figure 6. QIAquick® column in 2 ml collection tube

8. Apply the reaction mix to a QIAquick® column using a pipettor.

9. Centrifuge the QIAquick® column and collection tube for 30 seconds at 17,900 x *g* (13,000 rpm) (Figure 7).
   *Note: The linearized plasmid DNA is now bound to the column.

Figure 7. Tube placed in centrifuge and an example of centrifuge settings

10. Empty the collection tube into a liquid waste container.

Section V. Wash unbound molecules off of QIAquick® column

11. Add 750 µl Buffer PE to QIAquick® column (Figure 8) and place the column back into the 2 ml collection tube.

Figure 8. Buffer PE

   *Note: Buffer PE washes unbound molecules off of the column.
   *Note: The linearized plasmid DNA is still bound to the column.

12. Centrifuge 30 seconds at 17,900 x *g* (13,000 rpm).

13. Remove column and discard flow through that is in the collection tube into liquid waste beaker.
   *Note: The linearized plasmid DNA is still bound to the column.

14. Place QIAquick® column back into the 2 ml collection tube.

15. Centrifuge column and collection tube again for 30 seconds at 17,900 x *g* (13,000 rpm).

16. Place QIAquick® column in a clean 1.5 ml microcentrifuge tube (Figure 9) labeled with the sample name, your initials and the date.

Figure 9. Column in clean microcentrifuge tube

Section VI. Elute DNA

17. Add 30-50 µl of NF-H_2O water or Buffer EB to the center of QIAquick® column membrane.
    *Note: Whichever of these solutions you use, they will elute the DNA from the column.
    *Note: Whether you should use Buffer EB or water to elute depends upon what you are going to use the linearized plasmid for next. If you are going to use the linearized plasmid in an in vitro transcription reaction, for instance, then Buffer EB is a good choice.

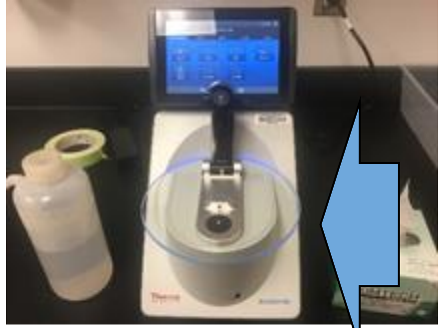
18. Centrifuge QIAquick® column and 1.5 ml microcentrifuge tube for 60 seconds at 17,900 x *g* (13,000 rpm).
    *Note: Linearized plasmid DNA is now in the solution in the 1.5 ml microcentrifuge tube.

Section VII. Use a Nanodrop™ Spectrophotometer to determine the concentration of the linearized DNA. This step is optional, as the amount of DNA should be the same as was added to the restriction enzyme digestion reaction, or 10 ug (see Step 1).

19. Clean Nanodrop™ with nuclease-free water and Kimwipes™.

20. Add 1 µl of solution used in step 17 (Buffer EB or NF-H_2O) to the Nanodrop™ and “blank” the Nanodrop™.

21. Wipe off Nanodrop™ with wet Kimwipe™ and add 1 µl of your linearized plasmid DNA sample.
22. Run the Nanodrop™ to obtain the concentration of your linearized plasmid DNA (Figure 10).



Place sample here

Figure 10. Arrow indicates where sample should be added to Nanodrop™

23. Check for linearization of your plasmid by running 1 µl of original plasmid and 1 µl of linearized plasmid in adjacent lanes on an 1% agarose gel. There should be a shift of the band on the gel when you compare the two lanes (Figure 11).

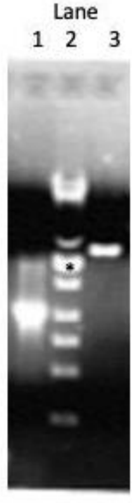


Figure 11: DNA gel comparing plasmid before (lane 1) and after (lane 3) being linearized with a restriction enzyme. The linearized plasmid will appear larger (more base pairs) than the circular plasmid because the circular plasmid doubles back on itself and therefore runs through the gel faster. The bright lane in the middle (lane 2) is the molecular size markers, the * indicates 1500 base pairs.

24. Store linearized plasmid at -20°C.

Cited References

1. Qiagen. 2020. QIAquick® Spin Handbook. Available from: https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/dna-clean-up/qiaquick-pcr-purification-kit/

Appendix D

Taqman® 1-Step qPCR (starting with RNA) Illustrated Protocol

Created by Jordyn Goddard, Espi Austvold and Ryan Steger

List of Source Protocols
Adapted from TaqMan® RNA-to-CT™ 1-Step Kit User Guide

Introduction
Quantitative PCR (qPCR) is a technique that allows you to monitor the progress of a PCR reaction in real time so that you can quantify the level of a sequence present in your sample. In this kit, the presence and quantity of your gene of interest is signaled by fluorescent reporter probes that fluoresce only when bound to a specific sequence. Based on the amplification rate of your targeted genetic sequences, you can compare the amount of fluorescence from your gene of interest to a control gene with known levels of expression to determine the relative quantity of that gene in your original sample. While similar to cDNA synthesis and cDNA qPCR, a One Step RNA to qPCR analysis allows you to perform a qPCR analysis directly from your RNA sample without the intermediate step of generating a separate cDNA sample. This process is faster and more efficient if you have a high volume of each RNA sample or are only interested in quantifying the expression of a few genes.

Reagent List
RNA sample on ice
Taqman™ Primers/Probes on ice
Nuclease-Free water (NF-H_2O)
Taqman™ RNA-to-Ct 1-Step Kit on ice

Equipment LIst
RNaseZAP
RNase Free p10 pipette tip boxes x2 (one unopened)
RNase Free p100 pipette tip boxes x2
RNase Free p1000 pipette tip boxes x2
RNase Free Microfuge tube rack
RNase Free PCR tube rack
RNase/DNase-free microfuge tubes in autoclaved beaker
p10 pipette
p100 pipette
p1000 pipette
MicroAmp Optical 96-Well Plate
MicroAmp Optical Adhesive Film Covers
Tape
Ice/Ice bucket

Safety: When carrying out this protocol, be sure to follow all safety and training guidelines set by your institution.

Section I. Calculating Your Volumes
You will be adding the following reagents to each of the wells of your plate that you intend to use. First you must calculate the proper volumes of RNA and NF-H_2O for you to use.

Reagents per well:
10 µl RT-PCR Mix
1 µl Primers/Probe
0.5 µl RT Enzyme Mix
___ µl RNA
\+ ___ µl NF-H_2O
20 µl Total Volume

1. First calculate the volume of template RNA required per well. This is based on the concentration of RNA in your sample. A volume of 1-5 µl is ideal; as is a total amount of 1000 ng RNA; therefore, RNA concentrations between 200-1000 ng/µl are needed.

To calculate:
Using the template RNA concentration (ng/µl), set up an equation to calculate the volume of µl required to get 1000 ng of RNA:

$$\frac{Y\ ng}{1\ \mu l} = \frac{1000\ ng}{X\ \mu l}$$

Y = concentration of RNA sample
X = volume you will use in reaction

Example:

$$\frac{295.5\ ng}{1\ \mu l} = \frac{1000\ ng}{X\ \mu l} \qquad X=3.4\ \mu l$$

This means it would take 3.4 µl of your sample to get 1000 ng of RNA.

2. Calculate the volume of NF-H_2O required per well so that each well has a total of 20 µl.

To calculate:
Subtract the volume of RNA per well and the volume of other reagents from 20 µl to determine how much water will be added to each well.

The combined volume of the other reagents is 11.5 µl:

10 µl RT-PCR Mix
1 µl Primers/Probe
0.5 µl RT Enzyme Mix
11.5 µl total volume

Equation:

20 µl - 11.5 µl other reagents - X µl of RNA sample per well = Z µl of water per well

Example:
20 µl - 11.5 µl other reagents - 3.4 µl of RNA sample per well = 5.1 µl of water per well
This means that you would add 5.1 µl of NF-H_2O to the well for that particular sample.

*Note: You will be using no template controls that purposefully exclude the RNA sample, these wells will require 8.5 µl of water each. Example: 20 µl - 11.5 µl = 8.5 µl

3. Repeat Steps 1-3 for all RNA samples you intend to use.
*Note: Your RNA samples will likely be of varying concentrations and therefore varying volumes will be needed. Keep track of which volumes correspond to which sample.

Section II. Setting up Wells

4. Prepare a template of your 96-well plate like the one below. A full size (8 x 12 wells) empty template is attached for your use. In this example, the table is set up to investigate two genes, Eya2 and Eya3, in two RNA samples (RNA 1 and RNA 2). 18S is an endogenous gene that is typically expressed at similar levels between samples and it serves to ensure that a consistent amount of sample is utilized in every well. Primers and probes are required for each of the genes including the controls. A no template control is a sample without any RNA. This is included to check for nucleic acid contamination of any reagents/equipment.

Table 1: Template of 96-Well Plate Setup

	Sample 1: RNA 1			Sample 2: RNA 2			Sample 3: No Template Control		
Gene 1: Eya2	Eya2 RNA1	Eya2 RNA1	Eya2 RNA1	Eya2 RNA2	Eya2 RNA2	Eya2 RNA2	Eya2 NF-H_2O	Eya2 NF-H_2O	Eya2 NF-H_2O
Gene 2: Eya3	Eya3 RNA1	Eya3 RNA1	Eya3 RNA1	Eya3 RNA2	Eya3 RNA2	Eya3 RNA2	Eya3 NF-H_2O	Eya3 NF-H_2O	Eya3 NF-H_2O
Control: 18S	18S RNA1	18S RNA1	18S RNA1	18S RNA2	18S RNA2	18S RNA2	18S NF-H_2O	18S NF-H_2O	18S NF-H_2O

*Note: Make sure you have DNase/RNase free materials and use RNA Zap to clean surfaces prior to continuing the procedure.

5. Prepare the master mix by combining the RT-PCR Mix and RT Enzyme Mix. The volume of master mix required will depend on how many wells you are planning on using.

*Note: Generally, reagents can be added to a master mix if they appear in the same ratio in all of the wells. This allows for consistency between wells and more efficient pipetting.

<u>To calculate:</u>
Multiply the volume of reagent required per well by the number of wells plus a few extra so that you have a slight excess of master mix in case of inefficient pipetting.

Equation:
10 µl RT-PCR Mix x (# of wells + ~3) = <u>µl total volume of RT-PCR Mix</u>
0.5 µl RT Enzyme Mix x (# of wells + ~3) = <u>µl total volume of RT Enzyme Mix</u>

Example for 27 wells:
10 µl RT-PCR Mix x (30) = <u>300 µl of RT-PCR Mix</u>
0.5 µl RT Enzyme Mix x (30) = <u>15 µl of RT Enzyme Mix</u>

Section III. Loading Wells

6. Place your 96-Well plate in a PCR tube rack (Figure 1). Make sure that well "A1" is positioned in the upper left corner.

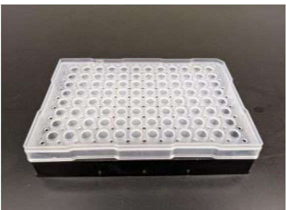


Figure 1. Example of 96-Well plate in a PCR tube rack

7. Pipette 10.5 µl of your master mix into each of the wells you plan to use (Figure 2). Make sure to pipet directly into the bottom of each well.

*Note: It is very important to keep track of which wells you have already pipetted into. One way to do this is to check off each reagent in your template as you add them.

Figure 2. Example of 10.5 µl in a p20 (left) and example of pipetting (right)

8. Pipette 1 µl of the correct primers (for your genes of interest) in each well by row according to the template. Check off each reagent in your template as you add them.
*Notes:

- When expelling these make sure to submerge the pipette tip in the master mix in each well. Only expel to the first resistance point on the pipet to decrease bubble formation.
- Make sure to get a new tip for each well.

9. Pipette the calculated volumes of RNA samples into each well according to the template. Check off each reagent in your template as you add it.
*Notes:

- The no template control (NTC) wells will NOT have any RNA in them, instead they will get NF-H_2O (see Step 10).
- Make sure to get a new tip for each well.

10. Pipette the calculated volumes of NF-H_2O into each well by column.
*Notes:

- Pipet 8.5 µl of NF-H_2O into each of the NTC wells.
- Make sure to get a new tip for each well.

11. Seal the plate using the adhesive film cover (Figure 3):

a. Peel back the white center layer from the clear adhesive film. This center region will be clear and there should be two white non-adhesive ends for handling.
b. Position the film directly over the whole plate, so it lines up with each edge.
c. Press down, sealing the plate from left to right, and from top to bottom with the sealing tool.
d. Use the corner of the tool to seal each well by running it down each and every row and column.
e. Carefully tear off the white handles on each of the sides of the film.

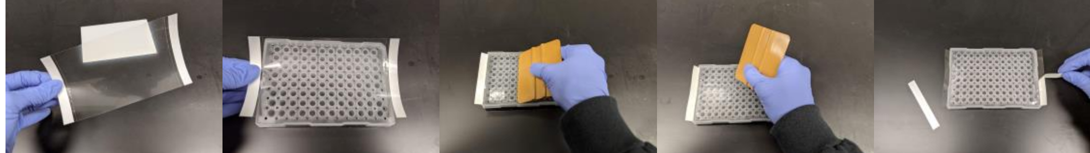


Figure 3. Example of Sealing Plate with Plate Film Cover, Steps 11a-e, left to right.

12. Once the plate is sealed, be sure to keep it even, do not tilt in any way. We want all of the solution at the bottom of the wells instead of on the sides. If needed, briefly centrifuge to get all of the solution to the bottom of each well.

Section IV. Running the qPCR Analysis

13. Turn on QuantStudio3 qPCR machine (the on/off switch is on the back right) and login to the computer attached to the machine.

14. Place the 96-well plate in the qPCR machine:

*Note: There are several different qPCR machine vendors and models. This protocol utilizes a QuantStudio3 qPCR machine and software. Your machine may require different steps.

a. Press button showing eject symbol on the touch screen -- this is located in the upper right-hand corner of the screen. The tray will then open.
b. Place the plate (without the PCR rack) with “A1” oriented in the upper left-hand corner on the tray.
c. Press the eject symbol button to close the tray.

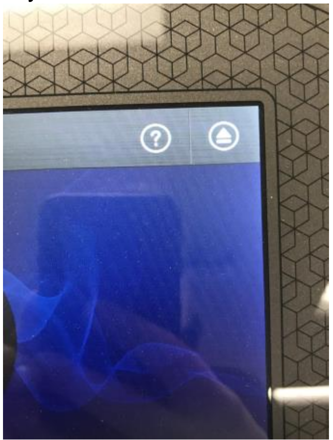
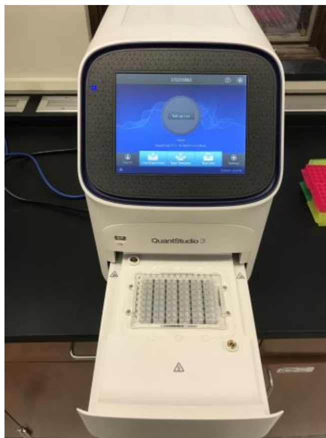



Figure 4. Eject Button on screen (left) and an open qPCR machine with the 96 well plate loaded (right).

15. Open “QuantStudio Design & Analysis Software”

16. Navigate to “Properties” tab
    a. Name the run
    b. Make sure that “Instrument Type” has “QuantStudio3 System” selected.
    c. Make sure that “Block Type” has “96-Well 0.1-mL Block” selected.
    d. Make sure that “Experiment Type” has “Comparative Ct ($\Delta\Delta$Ct)” selected.
    e. Make sure that “Chemistry” has “Taqman Reagents” selected.
    f. Make sure that “Run Mode” has “Standard” selected.

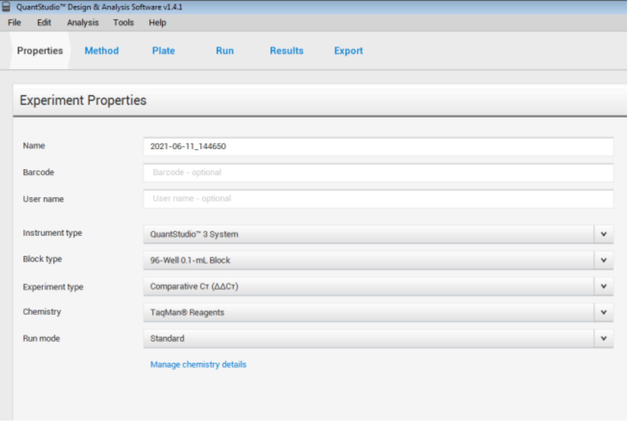


Figure 5. Example of the “Properties” tab

17. Under the “Method” tab set:
    48°C for 15 minutes
    95°C for 10 minutes
    95°C for 15 seconds
    60°C for 1 minute
    Set the last two steps to repeat 40 times.

18. Set the volume for 20 µl.

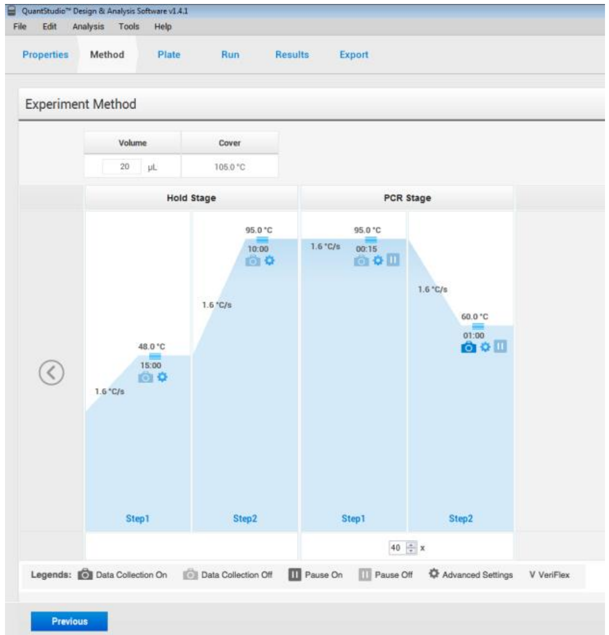


Figure 6. Example of the “Method” tab

19. Under the “Plate” tab:
    a. Highlight all wells that contain the same sample and enter a sample name.

    b. Highlight all wells that contain the same target gene and enter a target name.

    c. Highlight all NTC wells and under “Advanced Setup” tab on drop down menu and select “N” instead of “U” *Note: “N” stands for No Template Control, while “U” stands for Unknown Sample.

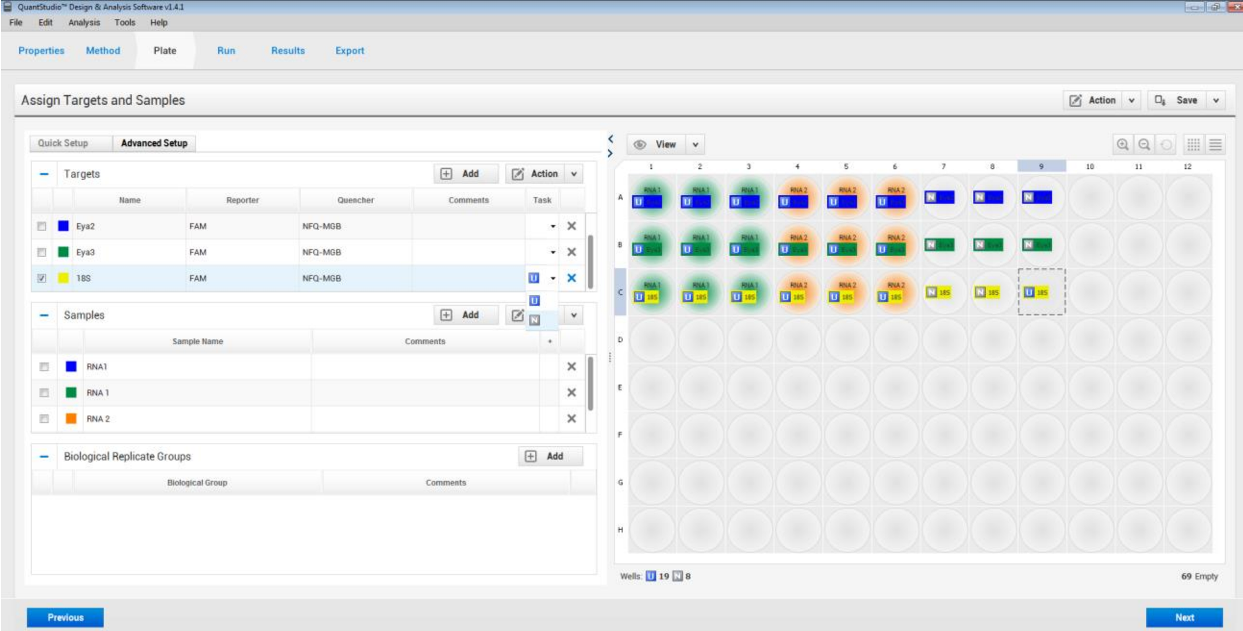


Figure 7. Example of the “Plate” tab

20. In the "Advanced Setup" tab, make sure the correct reporter is chosen for each target. The reporter type will vary depending on your probes. The reporter is the dye that creates a fluorescent signal that the qPCR reads. The reporter is attached to the 5' end of the probe. Check the container your probes came in to find this information.

Figure 8. Example of the tube for Eya2 - the reporter is "FAM"

21. In the "Advanced Setup" tab make sure the correct quencher is chosen for each target. The quencher type will vary depending on your probes. The quencher is attached to the 3' end of the probe, and prevents the reporter from releasing fluorescent signal when the probe is not bound to the target sequence.
Example: For Eya2/Eya3 the quencher is "NFQ-MGB."

22. In the "Quick Setup" tab under "Passive Reference" select "ROX". Passive Reference dyes are used to normalize for non-PCR related fluorescence signal variation.

23. In the "Quick Setup" tab select "Reference Sample".
Example: Select the RNA sample that is serving as a control.

24. In the "Quick Setup" tab select "Endogenous Control".
Example: 18S

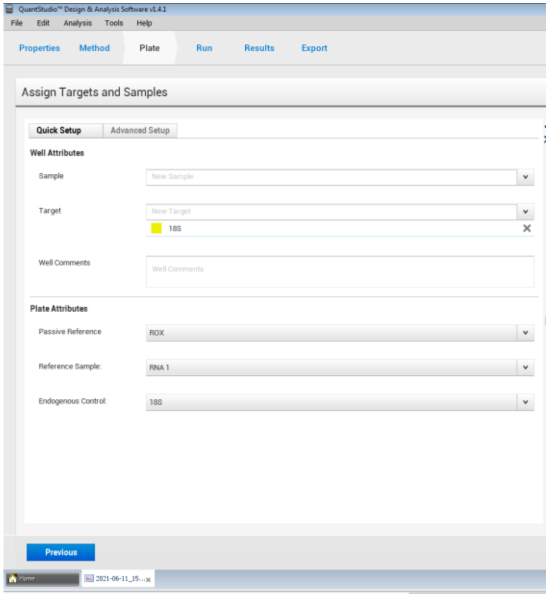


Figure 9. Example of the final "Quick Setup" (left) and "Advanced Setup" (right) views

25. Under the "Run" tab:
   a. Click the blue "start run" button.
   b. A drop-down menu will populate with the qPCR machine number, select this.
      *Note: This will take a few seconds to occur.

c. Confirm the file name and save the file.
d. The machine should start running and give a time estimate for the run.

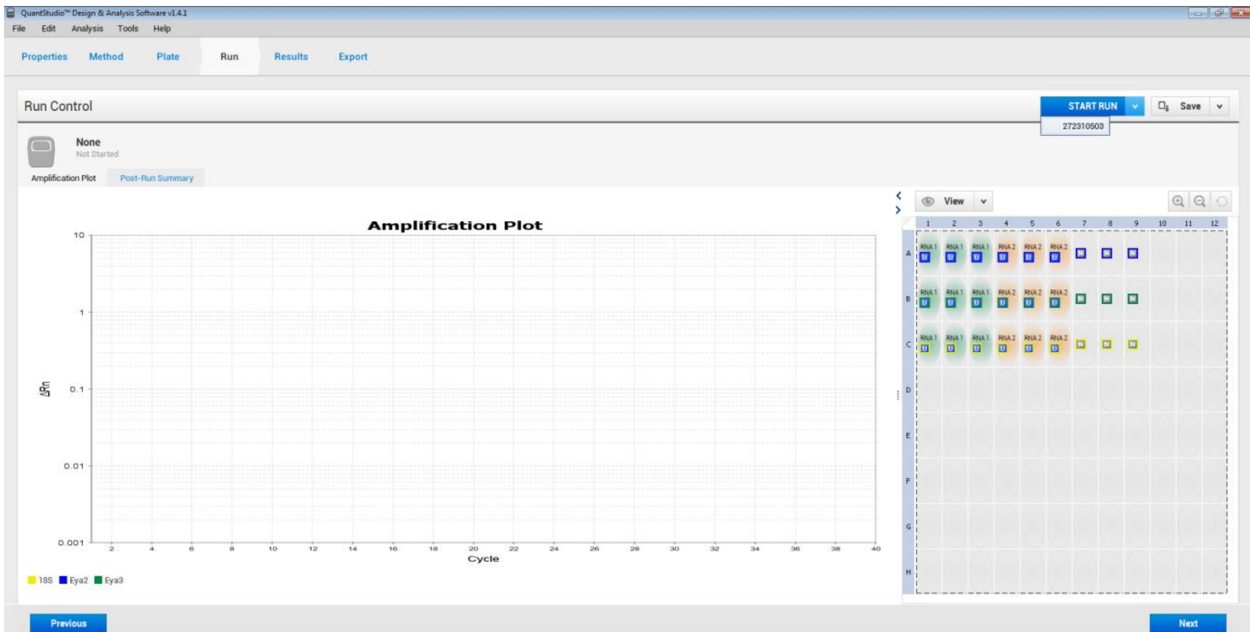


Figure 10. Example of qPCR machine number and 'Start Run' button

26. Once the run is complete:
    a. Make sure to remove the 96-well plate from the machine (using the same eject button).
    b. Save the file for later analysis. For backup, upload the file into your Thermo Fisher cloud account.
    c. Turn the machine off and shut down the computer.

Cited References

1. Life Technologies. 2012. TaqMan® RNA-to-CT™ 1-Step Kit Protocol. Available from: http://tools.thermofisher.com/content/sfs/manuals/4393463D.pdf

Template for 96-well plate

Genes of Interest	Sample 1:			Sample 2:			Sample 3:			Sample 4:		
Gene 1:												
Gene 2:												
Gene 3:												
Gene 4:												
Gene 5:												
Gene 6:												
Gene 7:												
Gene 8:												

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