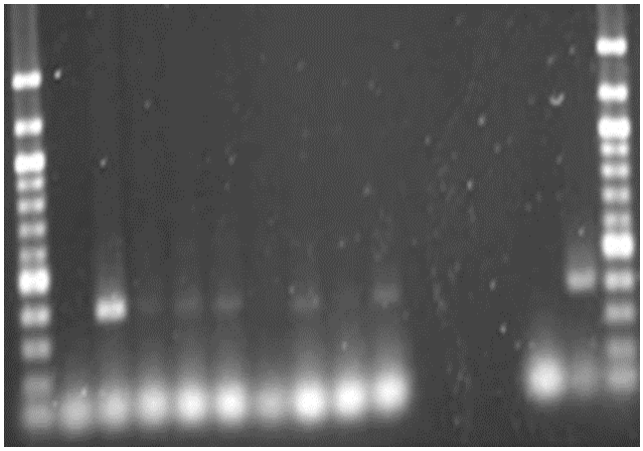
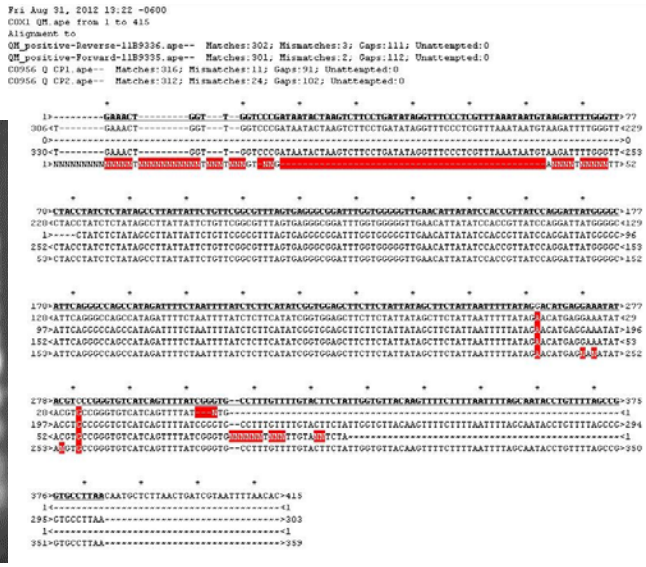
RECLAMATION

Managing Water in the West

Examination of Advanced Molecular Technologies

Research and Development Office
Science and Technology Program
Final Report ST-2016-6600-1





U.S. Department of the Interior
Bureau of Reclamation
Research and Development Office

September 2016

Mission Statements

The U.S. Department of the Interior protects America's natural resources and heritage, honors our cultures and tribal communities, and supplies the energy to power our future.

The mission of the Bureau of Reclamation is to manage, develop, and protect water and related resources in an environmentally and economically sound manner in the interest of the American public.

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The last ten years have seen an explosion of different molecular techniques and methods. These technologies can be used for a wide range of applications and research projects. The goal of this scooping project was to summarize some of these technologies and determine which ones could be of use at Reclamation and also if specialized equipment or reagents would have to be purchased. Reclamations Detection Laboratory for Exotic Species (RDLES) currently uses polymerase chain reaction (PCR), but there are additional technologies that can be used by RDLES to further Reclamations mission. Some of these technologies include other PCR techniques and methods, next generation DNA sequencing, and finally protein analysis methods.

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Notices
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Acronyms and Abbreviations

BSA- bovine serum albumin
cDNA- clonal deoxyribonucleic acid
CRISPR- clustered regulated interspaced short palindromic repeats
DNA- deoxyribonucleic acid
ELISA- enzyme-linked immunosorbent assay
IHC-immunohistochemistry
LAMP- loop mediated isothermal amplification assay
mRNA- messenger ribonucleic acid
NGS-next generation DNA sequencing
PCR- polymerase chain reaction
RDLES- Reclamation Detection Laboratory for Exotic Species
RNA- ribonucleic acid

Executive Summary

Recent advances in genetic analysis methods and instruments has started a revolution in the ways that researchers are able to design experiments and analyze samples. For example, these technologies can be used to conduct population studies, microbiome analysis, transcriptome analysis, and whole genome sequencing, and may be applied to a wide variety research questions. This is a scoping project to gather information on the current molecular biology technologies available and explore how these technologies could be applied to research being performed at Reclamation. Currently, Reclamations Detection Laboratory for Exotic Species (RDLES) performs conventional polymerase chain reaction (PCR) to detect the presence of organisms of interest in raw water samples. There are additional PCR and DNA sequencing methods available that could enhance the research being performed at RDLES. Also, there are protein detection methods, such as Western blotting and immunohistochemistry, which can be used to track the changes in protein concentration and even the location of proteins within a cell. Finally, the creation of transgenic organisms is of particular interest for the possibility of controlling the spread of invasive species. The speed of advancement in molecular methods that can be used to study and answer key questions is lighting fast, and this project is focused on the methods that can be applied and used by Reclamation to fulfill its mission goals.

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Introduction

The innovative technology polymerase chain reaction (PCR) is one of several technologies and techniques used by molecular biologists that have impacted a wide range of fields of research from medicine to ecological studies. From basic polymerase chain reaction tests to high throughput next generation sequencing (NGS) projects the techniques that have been developed over the last few decades can be used to address questions that field biologists and ecologists ask about the organisms that inhabit a place. The goal of this literature review is to summarize current molecular techniques that are available to Reclamation scientists.

This review will examine novel DNA amplification and sequencing technologies. There are also protein techniques such as enzyme-linked immunosorbent assay (ELISA), immunohistochemistry (IHC), and Western blotting that can be used to address a different set of questions. Finally, because it is an up and coming technology, CRISPR (clustered regularly-interspaced short palindromic repeats) will also be discussed. A brief description of each technique will be given, references provided, and costs associated with performing the method will summarized.

DNA Analysis Techniques

The invention of polymerase chain reaction (PCR) has impacted every person on earth. This technology gave researchers a way to amplify up a single gene of interest for analysis. From analyzing a small fragment of DNA this technology has expanded into many different directions. PCR can be used to analyze both DNA and mRNA expression levels. From conventional PCR many other PCR methods have been developed: quantitative PCR, real time PCR, and digital PCR.

The basic PCR reaction is comprised of three steps: denaturation, annealing, and extension. These three steps are repeated for 30-40 cycles to amplify the DNA fragment of interest. All PCR reactions also have common reagents that include: a buffer, dNTP's, primers, and DNA polymerase. There are additional reagents that can be added to PCR reactions, such as bovine serum albumin (BSA). Each reagent plays a role in the amplification process.

For all PCR methods, proper primer design is key for the assay to work correctly. If the primers are not specific to the gene of interest then the PCR will fail or amplify fragments other than the target of interest. There are some general rules for primer design. For example, the GC content should not be above 50% and the primer should not be able to form secondary structures [1]. Once the primers are designed, it is important to validate their specificity and sensitivity. For all PCR methods, the first step is to design primers that can robustly amplify the gene of interest.

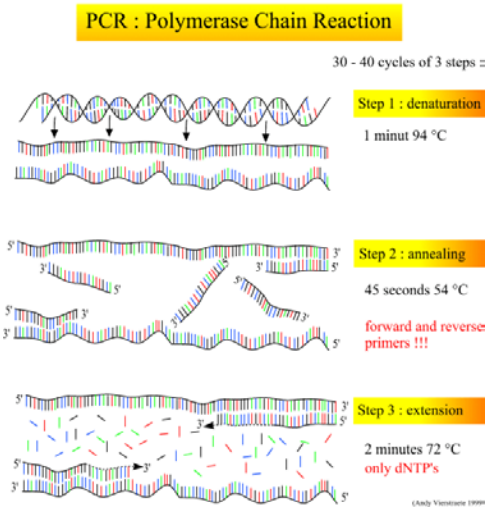

Figure 1: Basic PCR Reaction (From: Examination of advanced tech S&T report 9.14.2016-YP JK.docx accessed 8/19/2016)

Conventional PCR

In conventional PCR, the endpoint product is analyzed by gel electrophoresis to determine if the gene of interest was present or absent. This method is the one currently performed in the RDLES Laboratory. For these methods, the DNA in the reaction undergoes denaturation, annealing, and extension (Figure 1). Once the reaction is completed, the resulting PCR product is loaded onto an agarose gel for analysis to determine the presence or absence of a PCR product. There are many different types of electrophoresis methods that can be used for DNA, RNA, and protein analysis. The main one used for DNA analysis is agarose gel electrophoresis. In this method, the samples are loaded onto a gel, and then an electrical current is used to push the sample through the gel. The sample separates by size.

Cost: Purchase of the thermocycler, gel electrophoresis box and accessories, and gel reading apparatus

Ongoing costs:
PCR reagents: *Taq* polymerase, magnesium chloride, nucleotides (dNTP's), and primers
Gel reagents: gel running buffer, agarose, gel loading dye, and molecular weight marker

Quantitative PCR (qPCR)

Quantitative (qPCR) or real time PCR uses fluorescent dyes to monitor the production of PCR products as the PCR reaction is being carried out. Reverse transcriptase (RT-PCR) refers to the analysis of messenger RNA (mRNA) expression is samples. It should not be confused with real time PCR.

There are two different ways that these qPCR assays can be carried out. First, a non-specific dye can be used, such as SYBR green [2]. The second method

involves using a third internal primer that carries a fluorescent probe. When the DNA binds to this probe, it is released and measured by the instrument. When the signal become greater than the background it is possible to start to detect the samples. Both of these methods rely on the instrument to indicate when the signal is above the noise.

Cost: qPCR thermocycler (There are many types available on the market from companies such as BioRad, Qiagen, and Roche.)
Ongoing Costs: PCR reagents, fluorescent probes or dyes

Digital PCR (digital droplet PCR)

An up and coming PCR method is digital PCR (dPCR) (Figure 2). This method involves taking the DNA sample and partitioning it into many smaller reactions [3]. Each reaction cell contains only a few strands of DNA. As the PCR reaction is carried out cells that are positive for the gene of interest are detected by fluorescence analysis. After the analysis is done it is possible to get an absolute quantification of the amount of final product that is made. There are only a few companies that are making these instruments now. Each company has set up slightly different chemistries and ways that for the samples to be analyzed. The cost of the instrument and then the ongoing costs for supplies is currently an issue for this method. Currently, the cost of the instrument is very high, thus if this method would be pursued it would be beneficial to find a collaborator who already has a dPCR instrument. The full potential of dPCR is just starting to be realized and as the cost of the instruments should start to decrease as more companies provide instruments with this technology and more users adopt the technique.

Cost: Digital Thermocycler
Ongoing Costs: Disposable cassettes for sample analysis

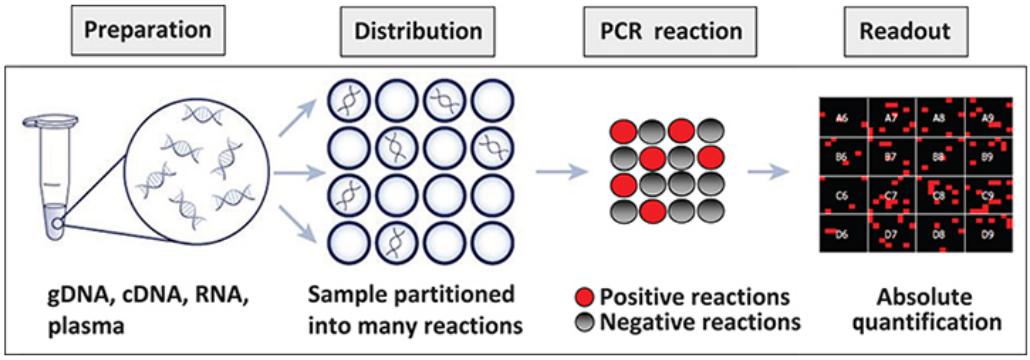

Figure 2: Digital PCR (From: https://www.thermofisher.com/us/en/home/life-science/pcr/digital-pcr.html)

Loop Mediated Isothermal Amplification (LAMP) Assay for the Detection of DNA

Unlike PCR methods, which require thermal cycling, LAMP, is an isothermic technique. By using a polymerase with high strand displacement activity, in addition to replication activity, target sequences can be ampified while the reaction is held at a constant temperature. Usually, four primer pairs are used for amplification of the target gene. There is software (Primer Explorer Version 3) that can be used to design the PCR primers. Having correctly designed primers for the LAMP assay is an important key to a successful amplification. The constant temperature means that a thermal cycler is not required. This eliminates the need for specialized equipment and enables researchers to simple technology like heat blocks. Instead, a heat block can be used to carry out the reaction.

One instrument has been built (Real Time Turbidimeter (LA-500) that amplifies and analyzes LAMP products. This instrument uses fluorescence to detect the LAMP product. In addition, other PCR instruments can be used for the amplification steps. Detection methods for LAMP include colorimetric assays or agarose gel assay to detect positive bands. Colorimetric assays produce a color change in the PCR tube that can show visually that there is a PCR product. This versatility in instrument and detection method makes this assay accessible to a wider range of users.

LAMP assays have been designed for the detection of a wide range of organisms, including the pathogenic nematode (*Strongyloides stercolalis*)[4], malaria [5], and leishmaniasis [6]. It has also been used to detect plant pathogens (*Xylella fastidiosa*) in New Zealand [7]. LAMP assays are also being used to detect genetically altered organisms, such as food crops [8]. This assay has been designed as a low cost method for DNA-based detection of infectious diseases in areas where having a thermocycler is not possible. Fact that is technique does not require a thermocyler means that it could be adapted for use in the field in Reclamation projects more readily than conventional PCR.

The following webpage has a full description of the LAMP amplification process: http://www.premierbiosoft.com/tech_notes/Loop-Mediated-Isothermal-Amplification.html (accessed 8/19/2016)

Costs: No specialized equipment, can use a heat block or thermocycler
Ongoing Costs: Enzymes and reagents can be higher than conventional PCR

DNA Sequencing

Following PCR analysis it is important for the research and detection analysis performed at RDLES to analyze the resulting PCR product by DNA sequencing, so that it is possible to determine the exact sequence. Sanger sequencing was the first method developed for DNA sequencing (Figure 3). Originally, sequencing reactions were carried out using radioisotopes. The resulting reactions were analyzed by running the sequencing reactions on a gel and then reading the gel to determine the DNA sequence. This was a labor intensive and expensive process. Today fluorescent tags are used to automate the sequencing process. The cost and

time required to perform sequencing has gone down significantly in the last few years.

Cost: Sequencing company charges for sample clean up and DNA sequencing A single tube reaction can cost as low as three dollars per reaction. The cost of this technique has decreased substantially over the last ten years.
Data Analysis: Sequencing results are analyzed using bioinformatics programs, such as NCBI BLAST. There are many free analysis programs available.

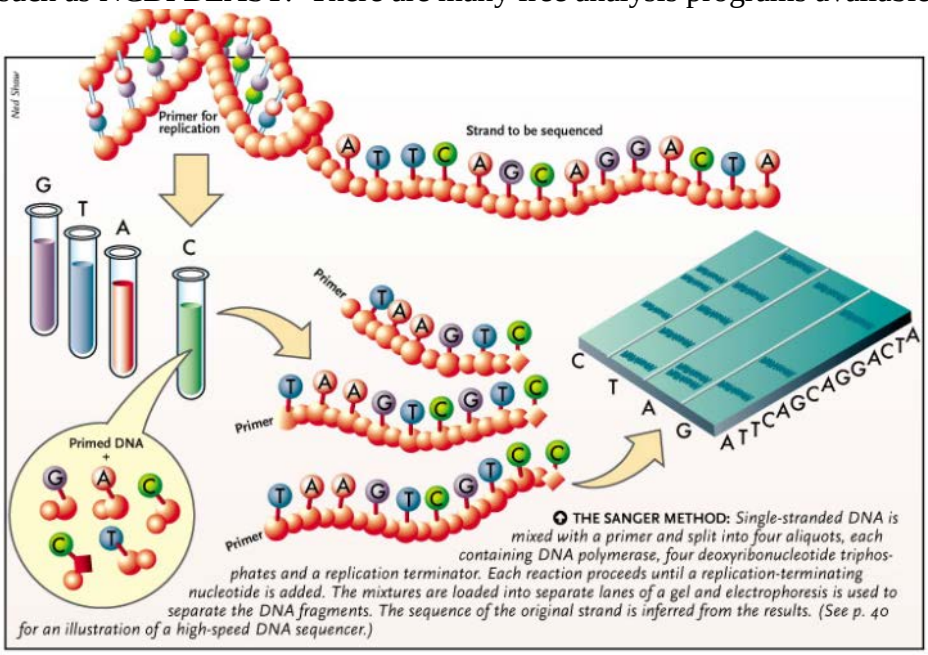

Figure 3: The Sanger sequencing method. (Edward Winnick, Sept 27, 2004, BioBusiness, The Scientist) From: http://www.the-scientist.com/?articles.view/articleNo/15939/title/DNA-Sequencing-Industry-Sets-its-Sights-on-the-Future/

Next Generation DNA Sequencing

The advent of next generation sequencing (NGS) has been a huge advance that is changing the way scientists approach experiments and ask questions. Instead of sequencing a single PCR product, in NGS all of the DNA sequences in a sample are analyzed. There are many decisions on the methods that will be used that have to be made (sample collection, DNA isolation, library creation, NGS instrument to use) to ensure that the results are as good as possible. The growth in the use of this technology for microbiome research can be tracked by the number of publications that use the word "microbiome". In 2001 there were only 74 articles, but by 2013 there were over 3,000 [9]. And that is only for microbiome studies, NGS has been used to sequence whole genomes [10]–[12]

Some projects relevant to Reclamation that could benefit from NGS include: fish stomach analysis, bird feces analysis, and environmental sample analysis for microbes. One of the advantages of NGS is that technique is a culture independent

method of analyzing a microbial community. Many microbes are hard or impossible to culture in the laboratory and NGS allows researchers to create a library of all the microbes present in a sample. The standard method is to analyze the 16S ribosomal RNA (rRNA) gene of bacteria [9]. Once the data is collected, identification of the bacteria present can aid in the determination of the biological function of the microbes in the environment [9].

There are several companies that produce different types of NGS instruments that have different chemistries, lengths of DNA that they can read, run time, and applications (Table 1) [13]. Rather than purchasing an NGS instrument, samples are usually sent to a commercial or university core facility for analysis. NGS instruments can be very expensive and creating the libraries is best handled by an expert who is performing these tasks on a regular basis. There have been many reviews published on NGS technology and methods. The following publications are a selection of these reviews: [10], [13]–[16], [11], [17]

Table 1: Common NGS instruments that are available (from [13]).

Table 1: Popular NGS platforms currently available in the market. The table shows the characteristic features of the high-end sequencing platforms and the recent "bench - top" platforms

High-end sequencing- Platform†	Sequencing chemistry	Read lengths/ through put	Run time	Template prep	Application
Roche 454 -Titanium FLX	Pyrosequencing	400 bp 400 Mb/run	10 hours	Emulsion PCR	Denovo WGS of microbes, pathogen discovery, Exome seq
Illumina/Solexa -HiSeq 2000	Reversible terminator chemistry	2×100bp 600 GB/ run (dual cell)	11.5 days	Solid-phase	Human WGS, exome seq, RNA-seq, Methylation
ABI/LifeTechnology-SOLiD 5550XL	Sequencing by ligation	2×60bp 15 GB/day	8 days	Emulsion PCR	Human WGS, exome seq, RNA-seq, Methylation
HelicosBiotechnologies	Reversible Terminator chemistry	25-55 bp 28 GB/run (avg)	>1 GB/hour	Single molecule	Human WGS, exome seq, RNA-seq, Methylation
Roche 454- GS Junior	Pyrosequencing	400 bp 50 Mb/run	10 hours	Emulsion PCR	Denovo WGS of microbes, pathogen discovery, Exome seq
Illumina/Solexa- MiSeq	Reversible terminator chemistry	2×150bp 1.0-1.4 Gb	26 hours	Solid-phase	Microbial discovery, Exome seq, Targeted capture
ABI/ Lifetechnology- Iontorrent	H+ Ion sensitive transistor	320 Mb/run	8 hours*	Emulsion PCR	Microbial discovery, Exome seq, Targeted capture

*Sample preparation – 6 hours, sequencing time – 2 hours, †Data shown here represent the highest figures currently available on the company website and is highly likely to change by the time this article is published

The final step in the NGS process is the data analysis. Large amounts of data are be produced in a single run, and understanding the best programs for the analysis of this data is key to ensuring that the results are valid. There are many different bioinformatics programs available for the analysis of the NGS data [17], and the company or core facility that performs the NGS run can usually help with this analysis. Taking the time to plan out an NGS experiment from start to finish is important to ensuring so that mistakes and issues can be avoided.

Costs: DNA extraction, library creation, use of NGS instrument for analysis, bioinformatics programs for data analysis

Protein Analysis Techniques

In addition to methods to analyze DNA there are many different methods that can be used to analyze proteins and enzymes. For example, a protein assay could determine the impact of a particular treatment on an invasive species to assess the amount of tissue damage that has been produced by the treatment.

ELISA (Enzyme-linked immunosorbent assay)

One type of protein assay is enzyme linked immunosorbent assay (ELISA) to analyze protein extracts. For this assay, a protein extract placed on a well that has been coated with an antibody of interest (Figure 5). By the end of the process the sample is analyzed at a specific wavelength to determine the concentration of the protein on interest.

Cost: Plate reader
Ongoing Costs: ELISA reagents

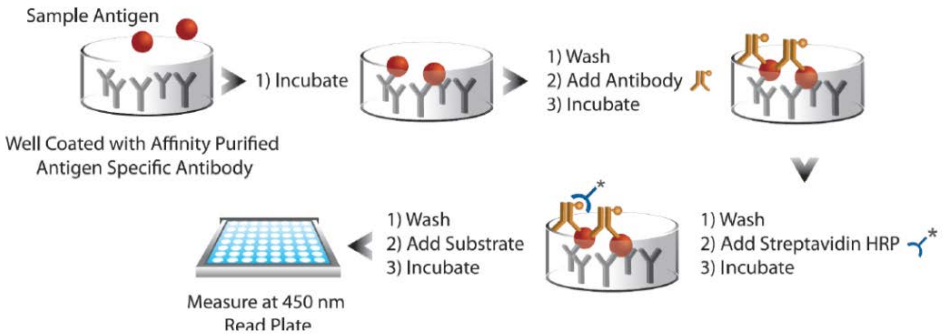

Figure 4: ELISA Assay (From http://static.enzolifesciences.com/fileadmin/redacteur/bilder/Platforms/Immunoassay_and_Assay_Development/Immunoassay_kits/2014-06-Immunoassay-ELISA-figure1.jpg (Accessed 8/22/2016))

Immunohistochemistry

This technique is used to analyze proteins using the microscope (Figure 6). For this method, the sample is placed on a microscope slide. If it is a tissue sample, it is first embedded in resin and then ultrathin sections are made that can be placed on a slide. Once the sample is on the slide, antibodies are used to detect a protein of interest. The primary antibody binds of the protein of interest, and then a secondary antibody is bound to the primary antibody that can be detected. Using a fluorescent microscope it is possible to visualize the location of proteins in interest in tissue.

Cost- equipment- microscopic and filters that can detect the secondary antibodies
Ongoing costs- antibodies (primary and secondary), buffers

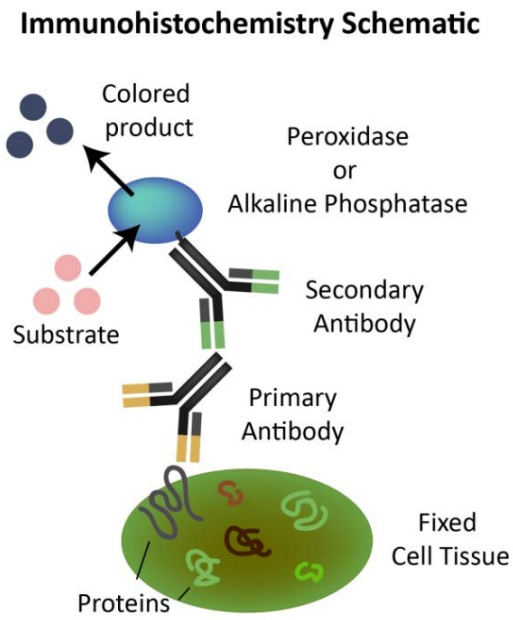

Figure 5: Immunohistochemistry Schematic (From http://www.rockland-inc.com/uploadedImages/ProductsStatic/Immunohistochemistry%20Schematic.jpg Accessed 8/22/2016)

Western Blotting

Western blotting is also a way of analyzing the proteins in a sample extract (Figure 7). For this method, an antibody is that is specific to the protein of interest is used to bind the protein. First, the protein in a sample is extracted. Then the sample is run on a denaturing gel to separate the proteins by size. Third, the proteins in the gel are transferred to a membrane. Finally, the membrane is stained using the antibody of interest. This method can be used to test for the presence and absence of specific proteins in a sample.

Cost: Equipment gel box, transfer apparatus
Ongoing costs: antibodies (both primary and secondary), buffers, extraction reagents

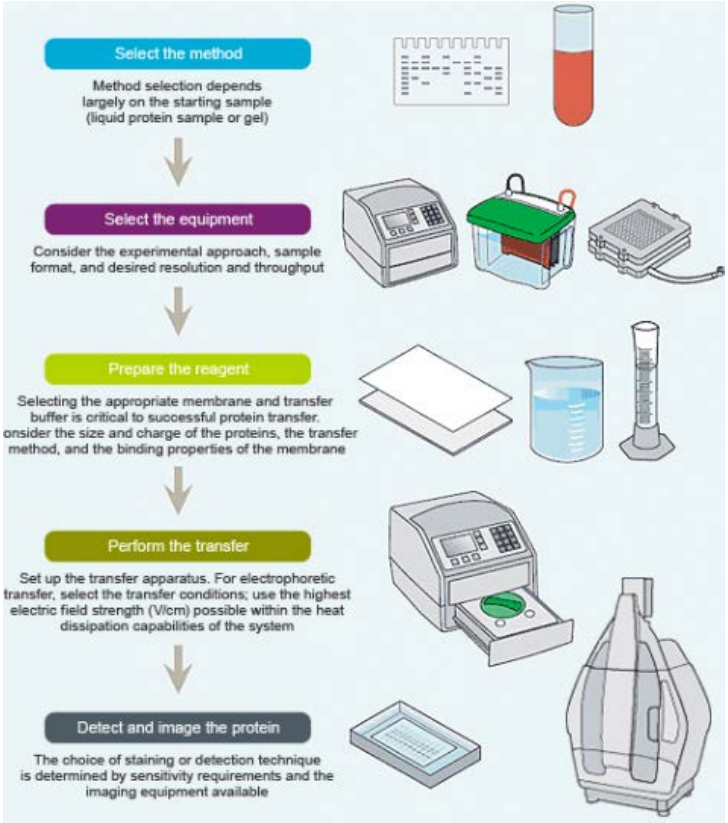

Figure 6: Western Blotting Work Flow (From http://www.bio-rad.com/webroot/web/images/lsr/solutions/technologies/protein_electrophoresis_blotting_and_imaging/western_blotting/technology_detail/western-blotting-workflow-western-blotting.jpg Accessed 8/22/2016)

Up and Coming Technologies

Genetic Manipulation

In the coming years there will be a great deal of research into the use of gene manipulation to control invasive species. The technology necessary for this type of research has only emerged in the last five years. In fact, the National Academy of Sciences has just issued a study on this subject, Gene Drives on the Horizon: Advancing Science, Navigating Uncertainty and Aligning Research with Public Values, 2016. This report gives a summary of current research and an overview of how genetic manipulation could be used to control some organisms. The main focus of the report was on clustered regulated interspaced short palindromic repeats (CRISPR) (Figure 7) and gene drive (Figure 8) technologies which are of major interest because of their ability to spread a gene throughout a population. The use of these technologies has serious ethical, moral, and regulatory questions associate with, and these issues will have to be addressed before this approach can be fully developed and implemented. For example, if it is possible to eliminate mosquitoes that spread disease, who has the right to release these modified organisms into the wild. Here is a list of several publications and reviews that provide background information on CRISPR and gene drive: [18]–[21]

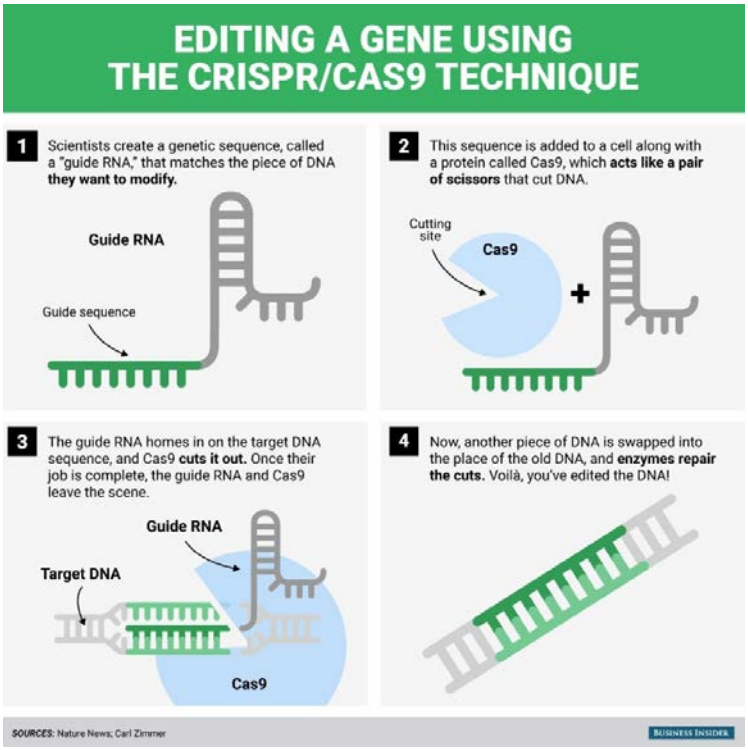

Figure 7: How CRISPR is able to edit a gene, (From http://static2.businessinsider.com/image/56392539bd86ef135c8bbe4d-1200-1200/crispr-infographic.jpg Accessed 8/22/2016)

The July 2016 Issue of National Geographic contained an article on genetic manipulation and CRISPR. These technologies are starting to be written about in the popular science press. Controlling the spread of malaria by CRISPR and gene drive could very easily happen in the next few years. There are two different approaches. First, to stop the spread of the parasite by making the mosquitoes resistant to the parasite. Second, to have all of the offspring be males and produce a daughterless community. This would lead to the extinction of the species of mosquitoes that carry malaria. These approaches could be applied to other diseases carried by mosquitoes, such as the Zika virus. The moral, legal, and scientific issues with releasing a transgenic organism into the wild and then driving the organism to extinction is one that society will have to address in the coming years.

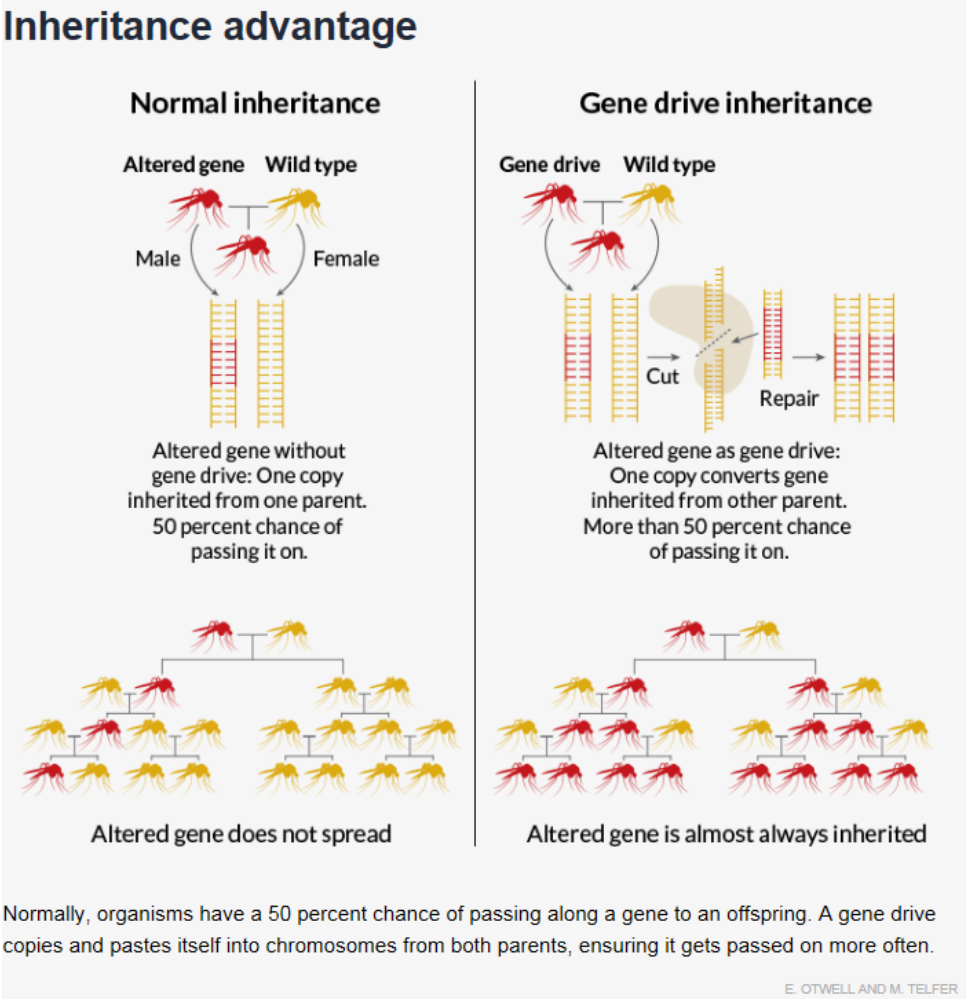

Figure 8: Gene Drive (From https://www.sciencenews.org/article/gene-drives-spread-their-wings, accessed 8/22/2016)

Summary

There are many different molecular biology techniques available that can be used by Reclamation researchers to address a wide range of questions. In 2016 an initial next generation sequencing project was started to survey the Lake Mead and Salton Sea microbiomes. A larger scale next generation survey of Reclamation waters will be undertaken in 2017 to better understand the impact of the invasive Dreissenid mussels on the microbiomes of several different water bodies. In addition, as the RDLES Laboratory moves forward in assay development, the LAMP assay will be explored as a possible amplification method for eDNA samples. Finally, a project was proposed for 2017 to explore the obstacles both technical and regulatory that would have to be overcome to create dreissenid mussels that have been modified by CRISPR for either produce daughterless broods of offspring or impact the mussels in some way that would decrease their numbers. The investigation performed for this scooping project has helped to guide future research in determining the best methods to use to answer a wide range of questions.

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