Development and Application of a Rapid, User-Friendly and Inexpensive Method to Detect *Dehalococcoides* spp. Genes in Groundwater

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Introduction

Technical Objective:

To develop rapid, sensitive and specific methods to quantify *Dehalococcoides* spp. genes from groundwater samples without DNA extraction

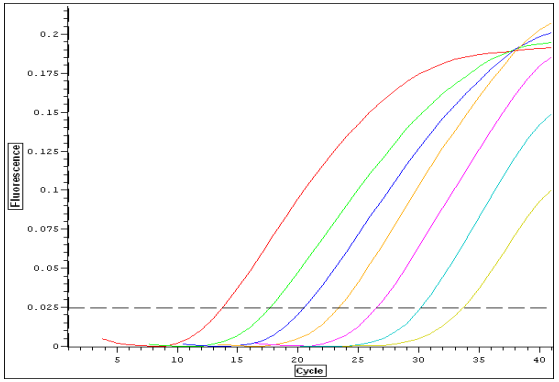
Key Components:

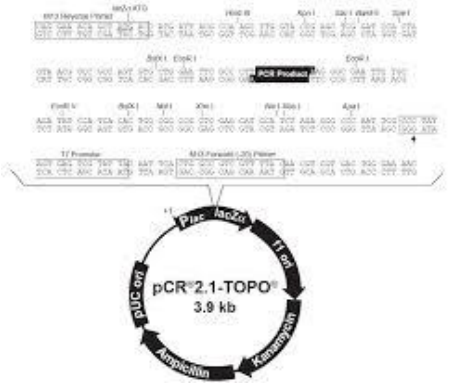
- ✓ Use loop mediated isothermal amplification (LAMP) as a technique for molecular detection
- ✓ Carry out validation with bioaugmentation cultures & groundwater samples
- ✓ Develop a LAMP based method which is low cost and easy to use
- ✓ Explore the development of field deployable approaches

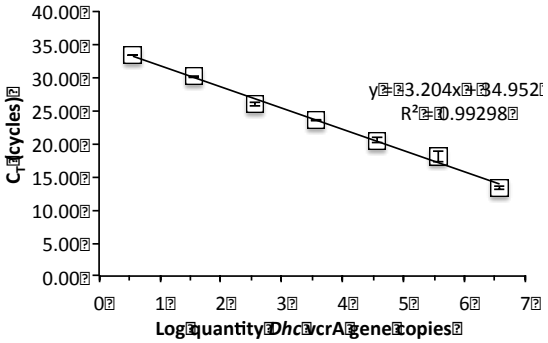
Introduction:

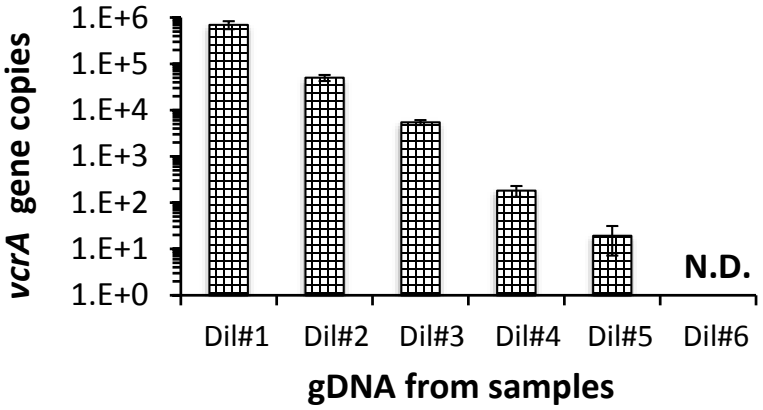
Conventional Detection of RDase Genes

- Typically, quantitative polymerase chain reaction (qPCR) is used for detecting and quantifying 16S rRNA or functional genes
- The gene of interest is cloned into pCR2.1 to make plasmid standards
- A standard curve with C_T values is plotted
- Environmental DNA is measured using a standard curve





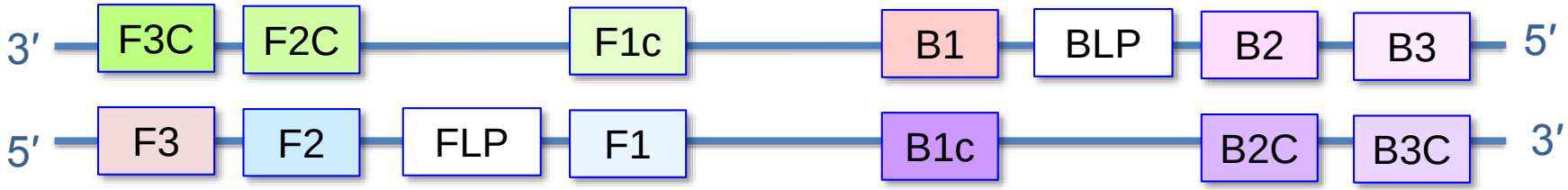




Introduction:

Loop Mediated Isothermal Amplification (LAMP)

Amplification creates stem loop structures with several inverted repeats of the target & cauliflower like structures with multiple loops



Forward Internal Primer (FIP) F1c F2

Backward Internal Primer (BIP) B1c B2

having both sense and antisense sequence helps in the formation of a loop

Forward Outer Primer (F3) F3

Backward Outer Primer (B3) B3

Forward Loop Primer (FLP) FLP

Backward Loop Primer (BLP) BLP

Introduction:

Applicability of LAMP for Development of Field Deployable Kits

- Amplification occurs at one temperature (isothermal), thermal cycler may not be needed
- High amplification efficiency, DNA is amplified 10^9-10^{10} times in 15 – 60 min.
- Direct amplification without DNA extraction for water samples
- Less sensitive to substances that inhibit PCR
- Six primers make LAMP highly specific
- Because of these advantages LAMP has been widely applied as a point of care molecular diagnostic tool for detection of a variety of templates

Step 1: Biomass Extraction

Direct cell extractions from groundwater

Step 2: Detection of template

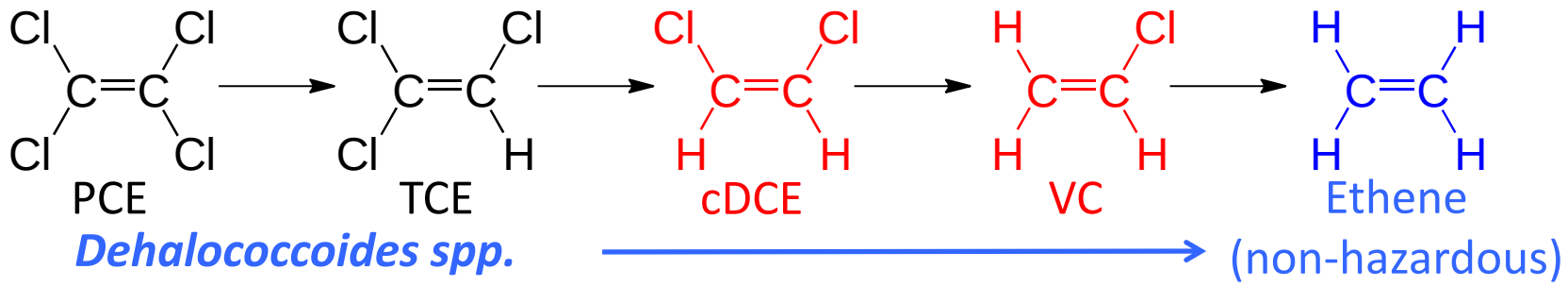
Visual detection with dsDNA binding dyes

Detection on real time thermal cyclers

Detection on proprietary microfluidic platforms e.g. Gene-Z

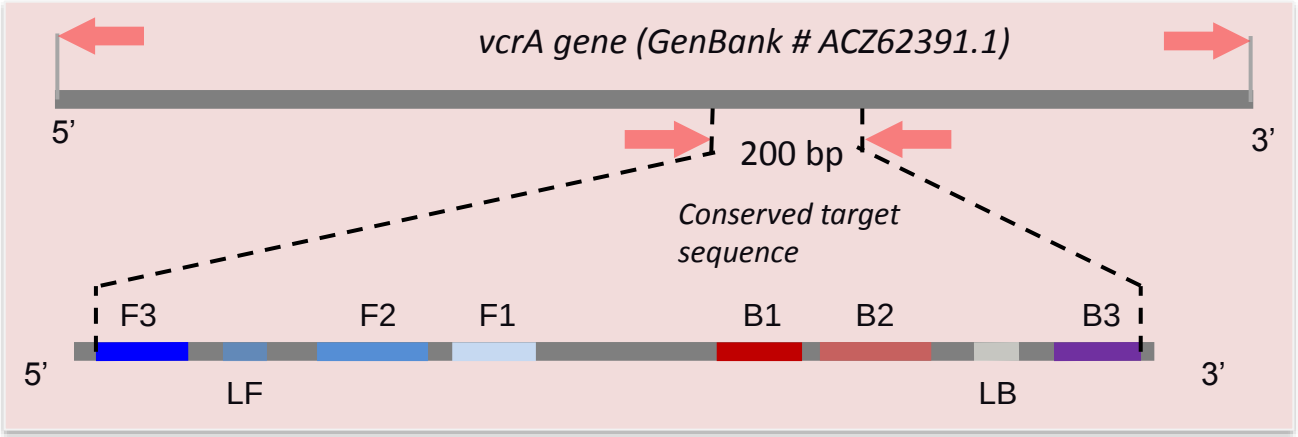
LAMP with Rdase genes

Primer Development



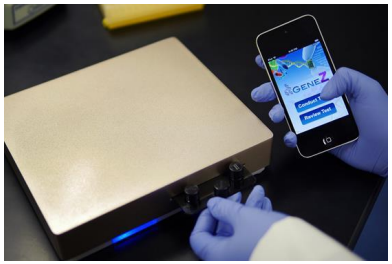
- Survival & activity is typically determined using qPCR targeting 16S rRNA or functional genes (RDase genes)
- Three LAMP primer sets specific to the *tceA, vcrA*, and *bvcA* genes were designed using Primer Explorer v4

LAMP primer designing software
PrimerExplorer



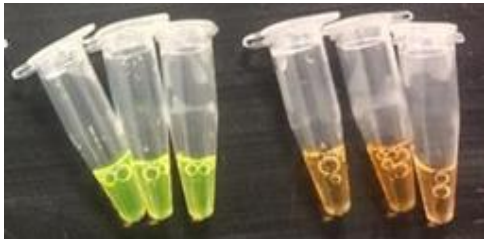
Three Study Questions

Q1. Is **LAMP comparable to qPCR** for DNA extracted from **cultures** or **groundwater**?



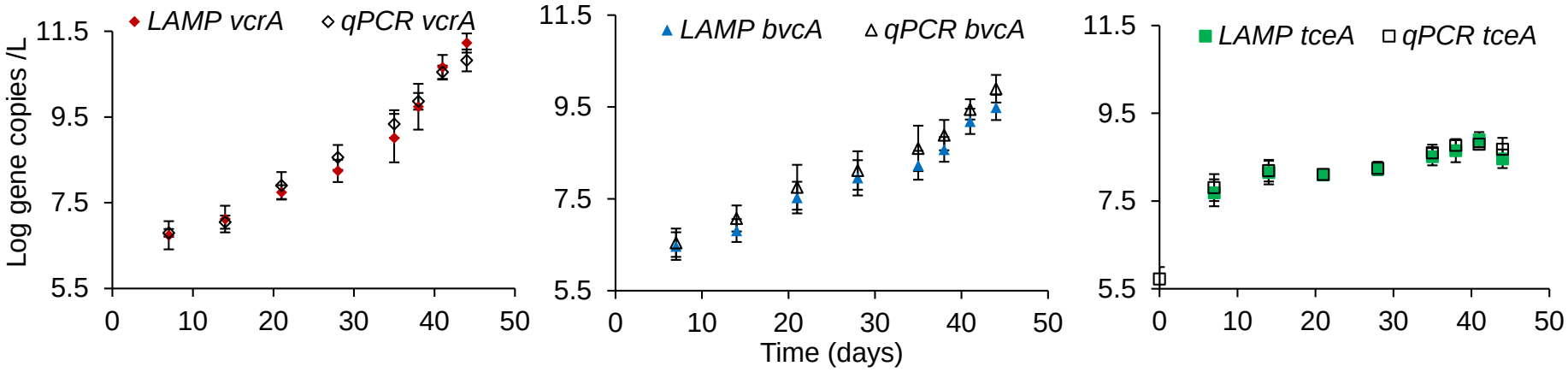
Q2. Is **LAMP with Gene-Z** comparable to **qPCR with a real time thermal cycler**?

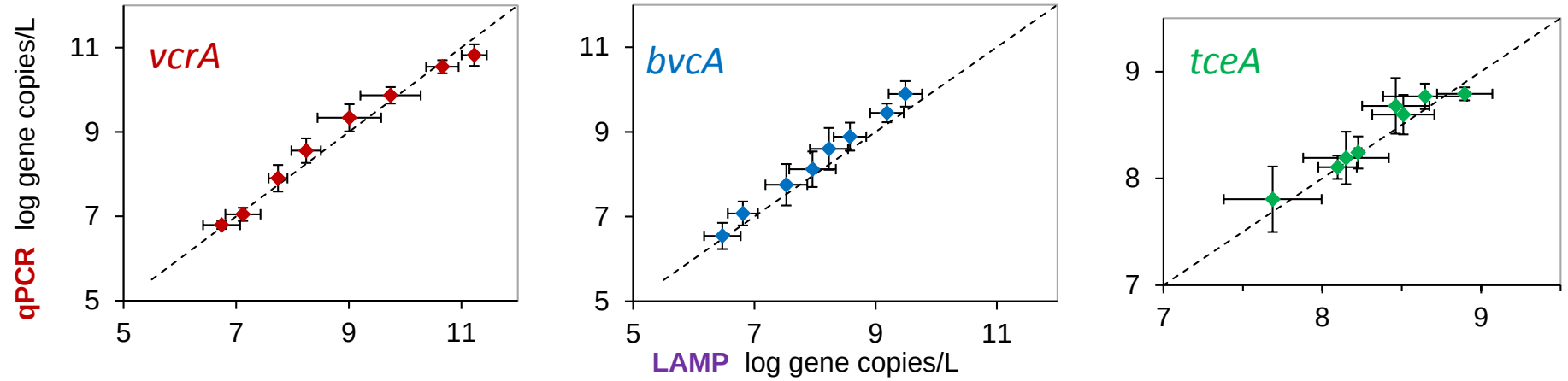
Q3. How viable are **visual based LAMP assays** on **cell templates** (no DNA extraction) for detecting RDase genes?



LAMP with Rdase genes

Comparing LAMP to qPCR with KB-1 DNA templates

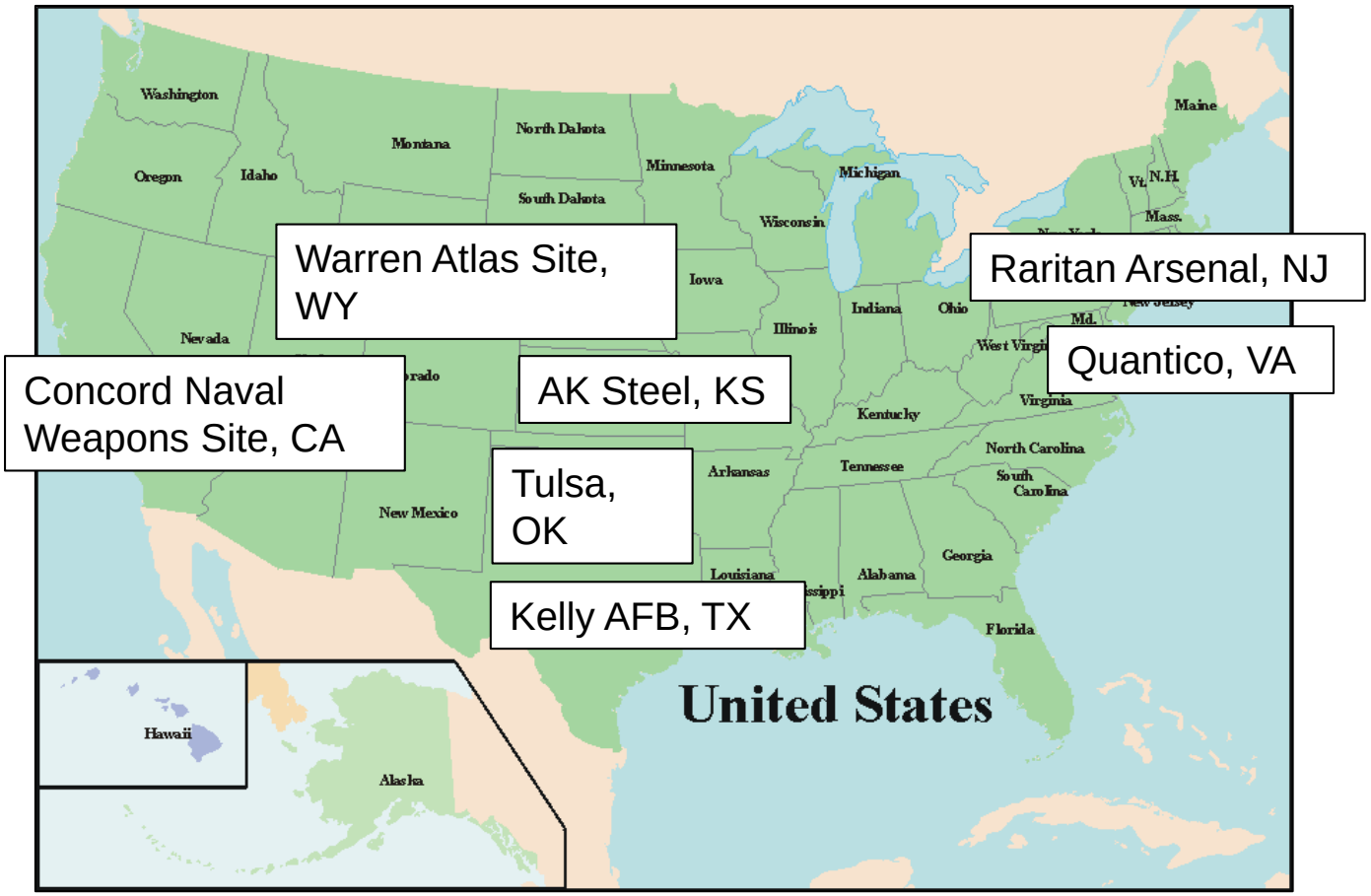




- LAMP primers were used to track the growth of *Dehalococcoides* spp. over one growth cycle of KB-1
- Quantification with LAMP was comparable to qPCR
- Similar results were observed with SDC-9

LAMP with Rdase genes

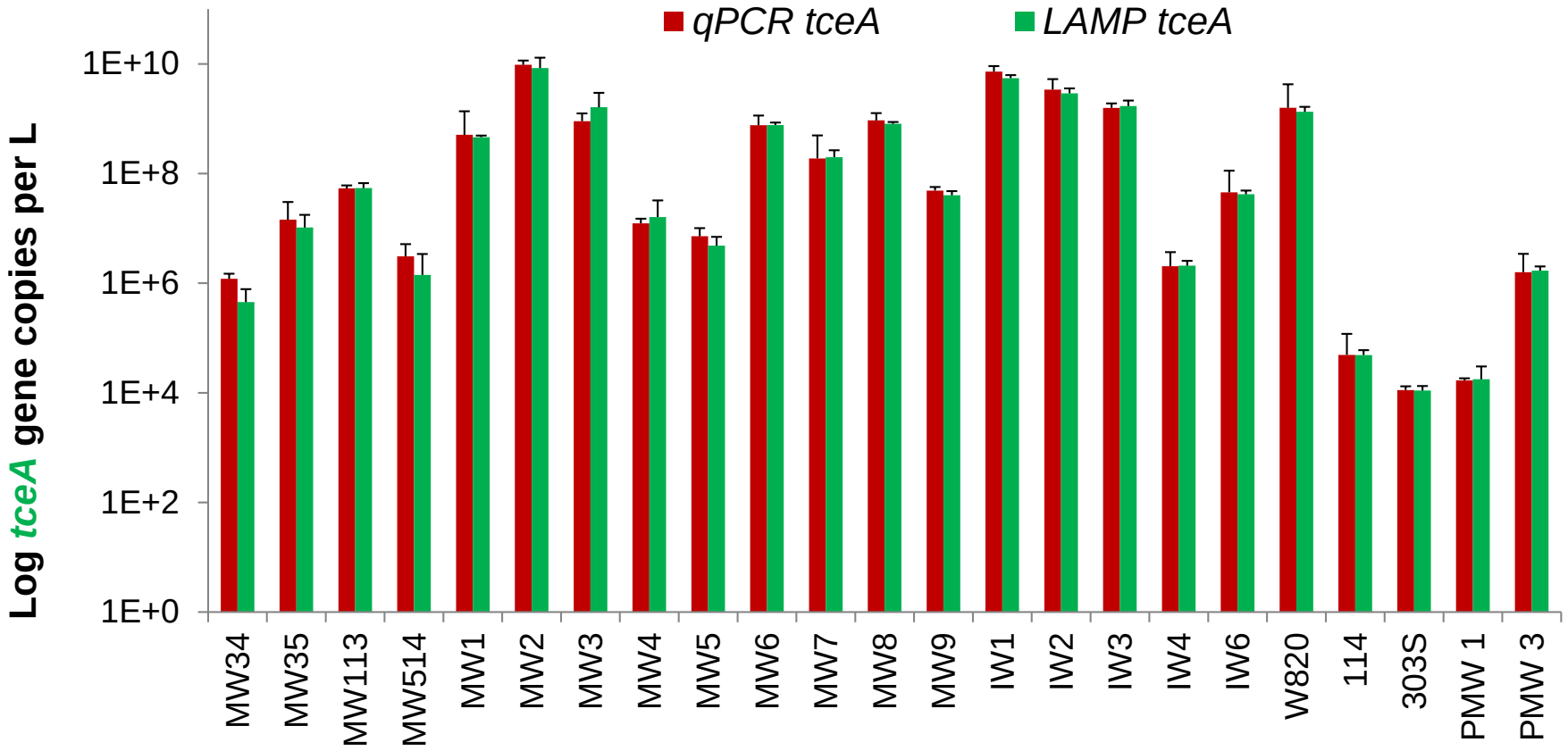
Comparing LAMP to qPCR with groundwater templates



- 27 groundwater samples from 7 sites were shipped to Michigan State University for analysis
- DNA extracts from groundwater samples were used as templates for comparing LAMP to qPCR

LAMP with RDase genes

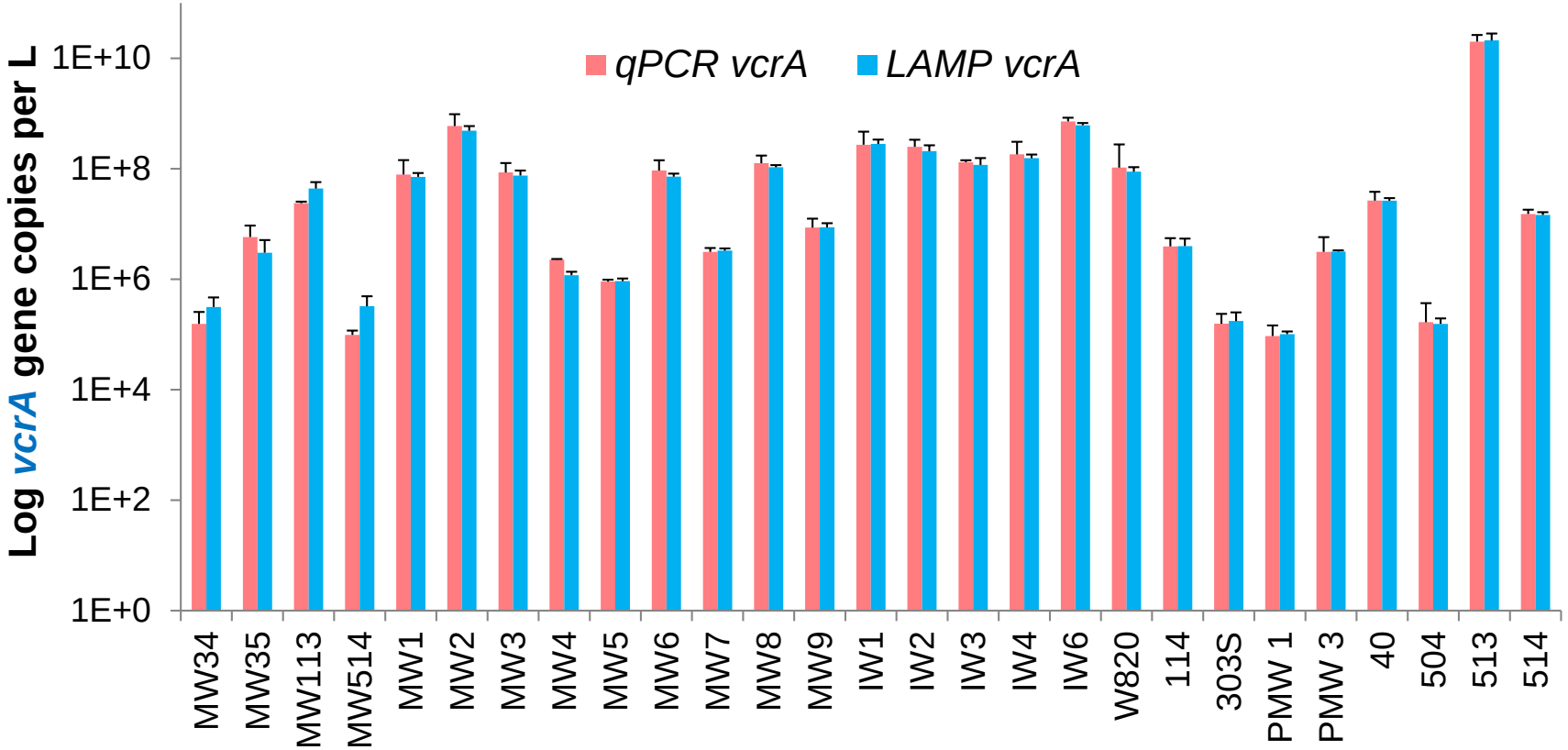
Comparing LAMP to qPCR with groundwater templates (tceA)



- qPCR & LAMP produced similar results for *tceA*
  - ✓ Similar results for different groundwater samples
  - ✓ Similar results over a range of concentrations
  - ✓ Replication was good between triplicate samples

LAMP with RDase genes

Comparing LAMP to qPCR with groundwater templates (vcrA)



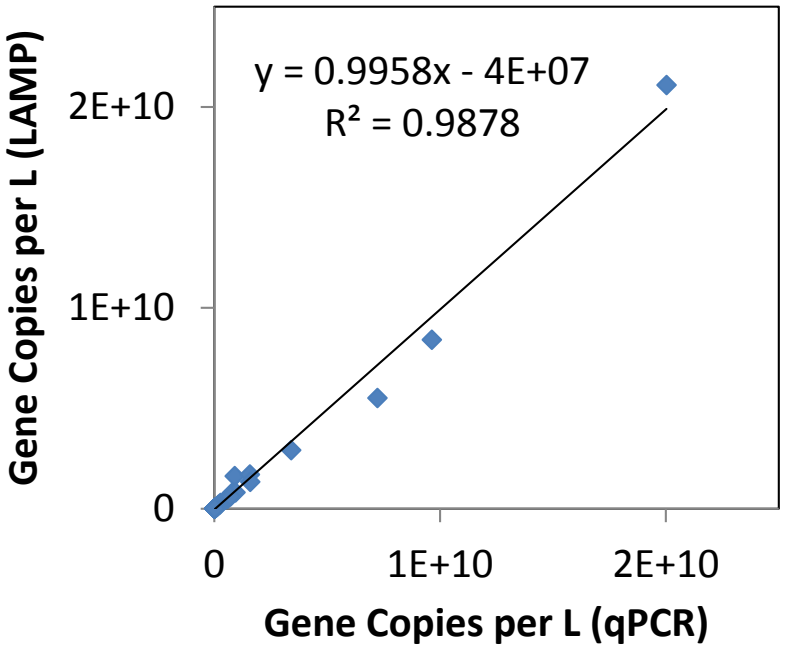
- qPCR & LAMP produced similar results for *vcrA*
  - ✓ Similar results for different groundwater samples
  - ✓ Similar results over a range of concentrations
  - ✓ Replication was good between triplicate samples

LAMP with RDase genes

Summary - DNA from Groundwater qPCR compared to LAMP

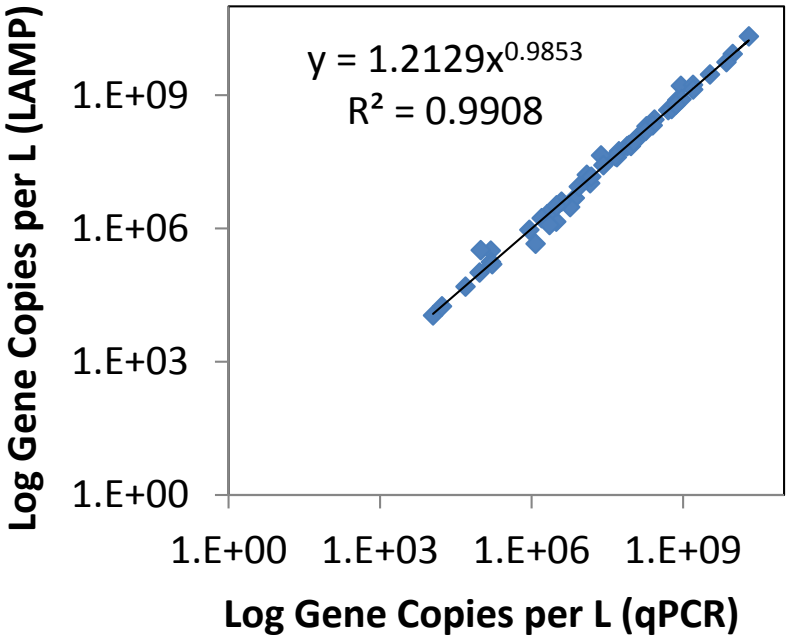
LAMP vs. qPCR (*tceA* & *vcrA*)

Arithmetic Scale



LAMP vs. qPCR (*tceA* & *vcrA*)

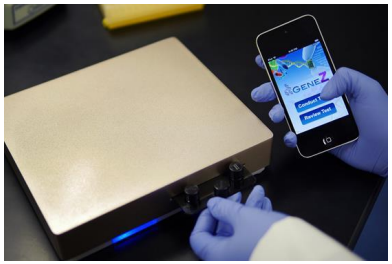
Log Scale



- qPCR & LAMP produced similar results for DNA extracted from cultures and from SDC-9 bioaugmented groundwater
- Quantification with LAMP was comparable to qPCR over a wide range of concentrations for *tceA* and *vcrA* genes when DNA was used as templates

Three Study Questions

Q1. Is **LAMP comparable to qPCR** for DNA extracted from cultures or groundwater?



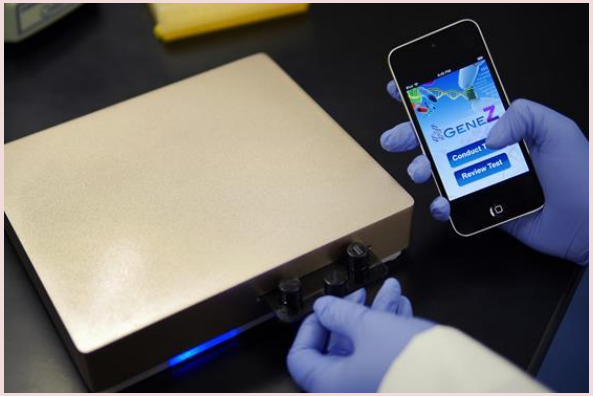
Q2. Is **LAMP with Gene-Z** comparable to **qPCR with a real time thermal cycler**?

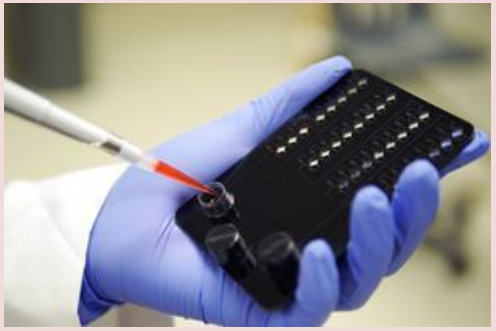
Q3. How viable are **visual based LAMP assays** on **cell templates** (no DNA extraction) for detecting RDase genes?

Alternate Platforms for Detection

Gene-Z Analyzer

- Gene-Z is an inexpensive handheld device developed by Dr. Hashsham's group for the parallel detection of pathogens
- It offers quantitative isothermal amplification based on LAMP and is operated using an iPod Touch
- Multiple LAMP assays can be performed on a microfluidic chip (made in house)
- The chip is loaded using a one step process for dispensing the sample
- Analysis of the sample usually takes less than two hours

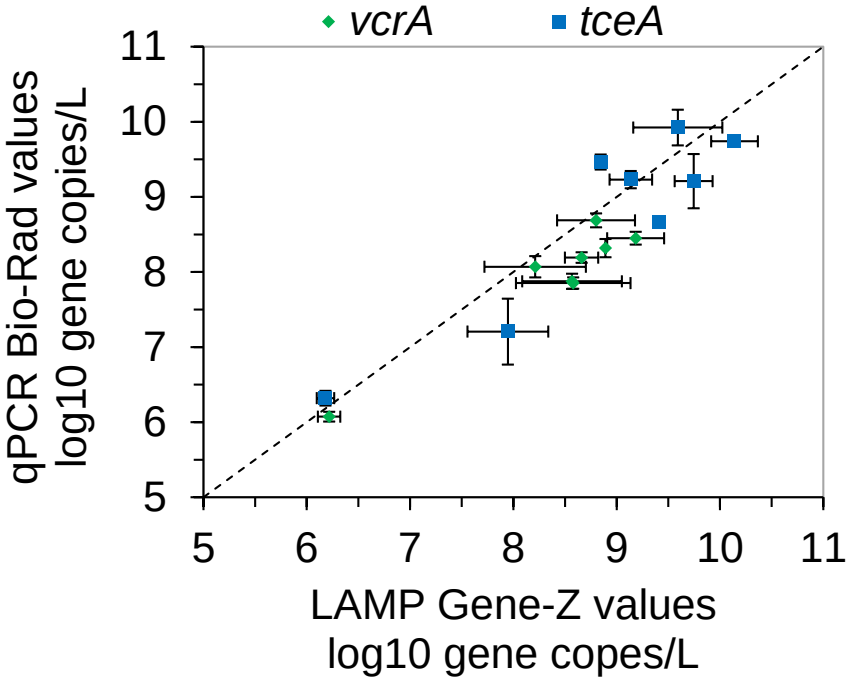




Alternate Platforms for Detection

Quantification in SDC-9 bioaugmented groundwater with LAMP

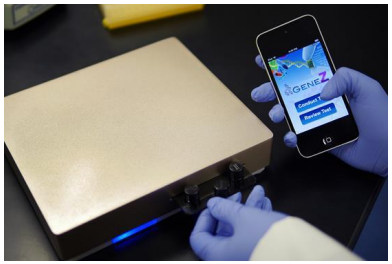
- DNA extracts from groundwater samples were used as templates to compare qPCR based quantification to quantification on the Gene-Z
- Quantification with LAMP Gene-Z was comparable to that of qPCR over a wide concentration range for both *tceA* and *vcrA* genes



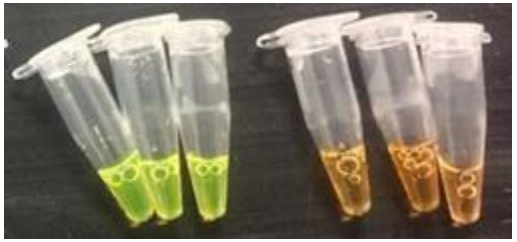
Kanitkar, Y. H.; Stedtfeld, R. D.; Steffan, R. J.; Hashsham, S. A.; Cupples, A. M., Development of loop mediated isothermal amplification (LAMP) for rapid detection and quantification of *Dehalococcoides* spp. biomarker genes in commercial reductive dechlorinating cultures KB-1 and SDC-9. *Applied and Environmental Microbiology* **2016**, *82:1799-1806*

Study Questions

Is **LAMP comparable to qPCR** for DNA extracted from cultures or groundwater?



Is **LAMP with Gene-Z** comparable to **qPCR with a real time thermal cycler**?



How viable are **visual based LAMP assays** on **cell templates** (no DNA extraction) for detecting RDase genes?

LAMP with RDase genes

Direct Cell Amplification on Real Time Thermal Cyclers

- Direct cell amplification is the addition of cells without DNA extraction to the LAMP reaction as a template for amplification
- It is very similar to colony PCR performed during M13 screen test
- Direct cell templates are often used with LAMP for rapid point of care diagnostics in human and veterinary medicine
- Direct cell and centrifuged cell templates were prepared from SDC-9 bio augmented groundwater and used to test LAMP

Creation of Direct and Centrifuged Cell Templates from Groundwater

Groundwater pre-filtered (5 µm), then passed through Sterivex filter

Add elution buffer (1000 µL), vortex to remove cells

Centrifuge, then resuspend the pellet

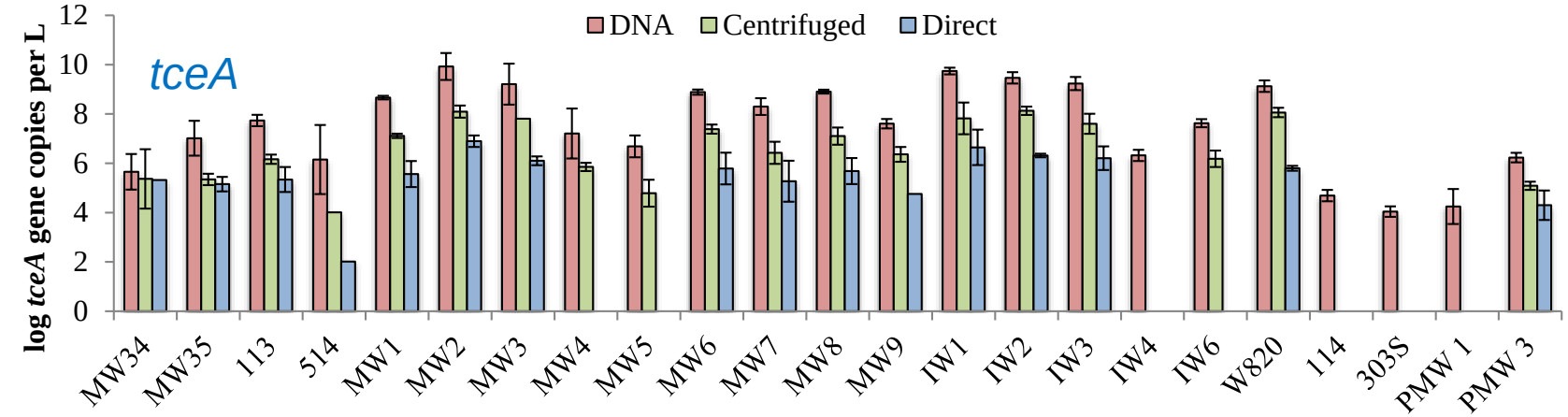
Direct Cell Templates

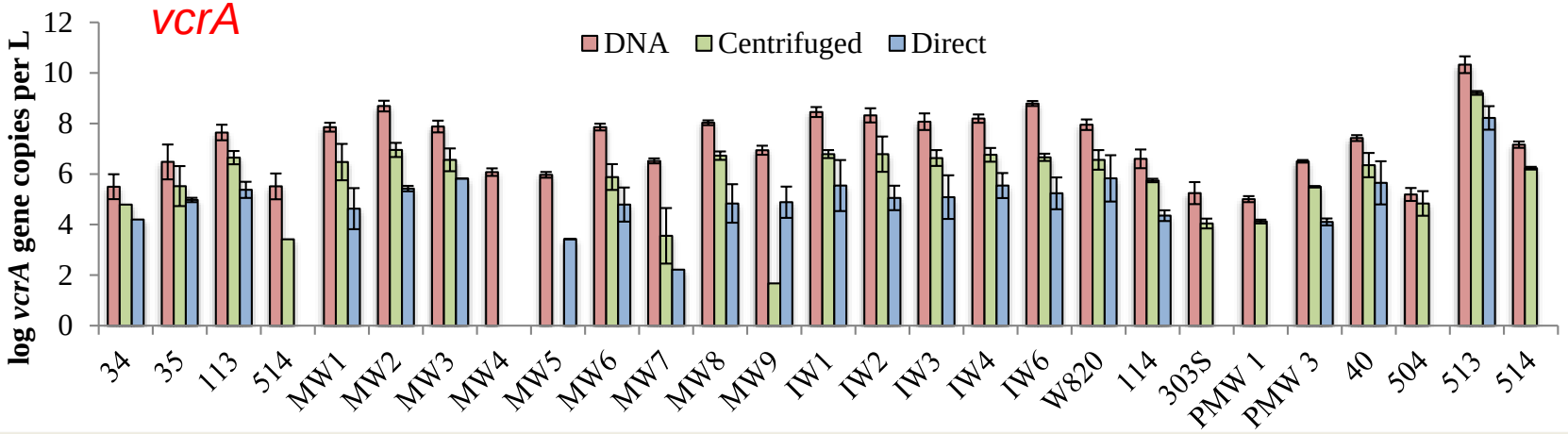
Centrifuged Cell Templates

Add 5 µL of sample to LAMP master mix

LAMP with RDase genes

Direct Cell Amplification on Real Time Thermal Cyclers



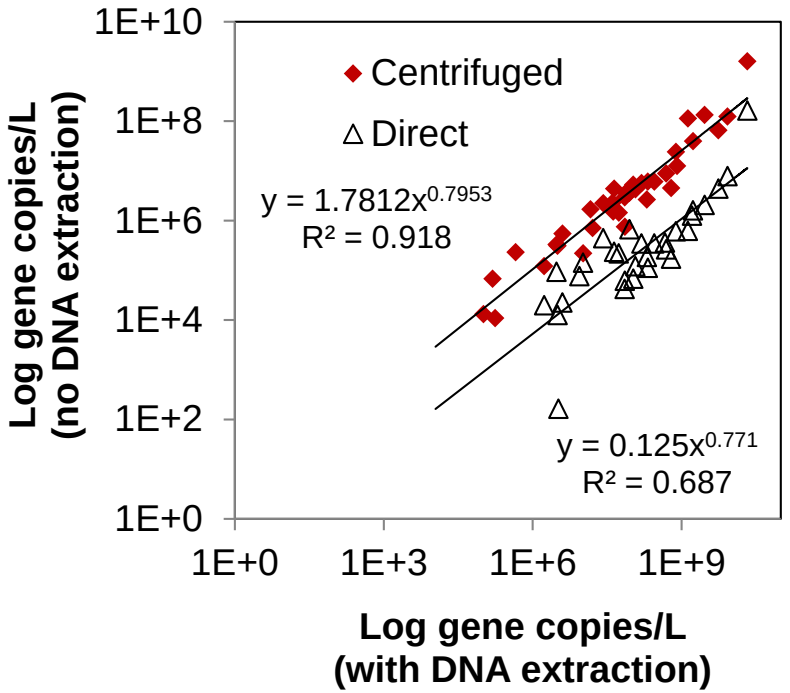


- Quantification with DNA templates > Centrifuged cell templates > Direct cell templates
- This trend was observed to be similar with both *tceA* and *vcrA* genes

LAMP with RDase genes

Summary - Amplification with Direct Cell Templates

- When biomass concentrations are increased using centrifugation, the observed gene copy/L concentrations also increase
- This suggests that observed gene copy/L concentrations are a function of eluted biomass concentration
- Quantification with centrifuged cell templates was more faithful to quantification with DNA templates



- Centrifuged cell templates had about half the gene copy concentration of DNA templates
- Direct cell templates had ~ 1/10 gene copy concentration of DNA templates

Alternate Platforms for Detection

SYBR Green LAMP assay

Pre-filter (5 µm) 100 mL, then use Sterivex filter

Elution buffer (1000 µL), then vortex & remove cell

Centrifuge, then resuspend pellet

Add 5 µL of sample to LAMP master mix, and then move to water bath (1 hr, 63°C)

Add SYBR Green

Orange: Control

Three Green: >1.8 X 10^5 *vcrA* per L

Kanitkar Y.H., Stedtfeld R.D., Hatzinger P., Hashsham S.A., Cupples A.M. (In press) . Development and application of a rapid, user-friendly and inexpensive method to detect *Dehalococcoides* spp. reductive dehalogenase genes from groundwater. Applied Microbiology and Biotechnology

Alternate Platforms for Detection

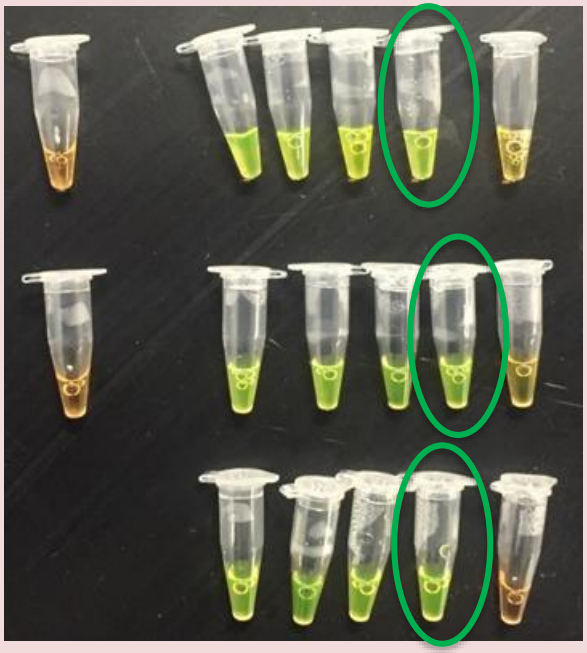
Detection Limits of SYBR Green LAMP Assay

- Detection limits of the SYBR Green LAMP assay when plasmid DNA is used as a template were evaluated
- A five fold 10X dilution series of *tceA*, *vcrA*, and *bvcA* genes were used as templates
- SYBR Green LAMP assay was able to detect ~100 *vcrA* gene copies per reaction when plasmid DNA was used as a template for amplification
- Similar results were observed for *tceA* and *bvcA* genes

Detection limits with plasmid DNA (vcrA gene)

Controls *Triplicates of a dilution series*

10^5 10^4 10^3 10^2 10



Dilution series 1

Dilution series 2

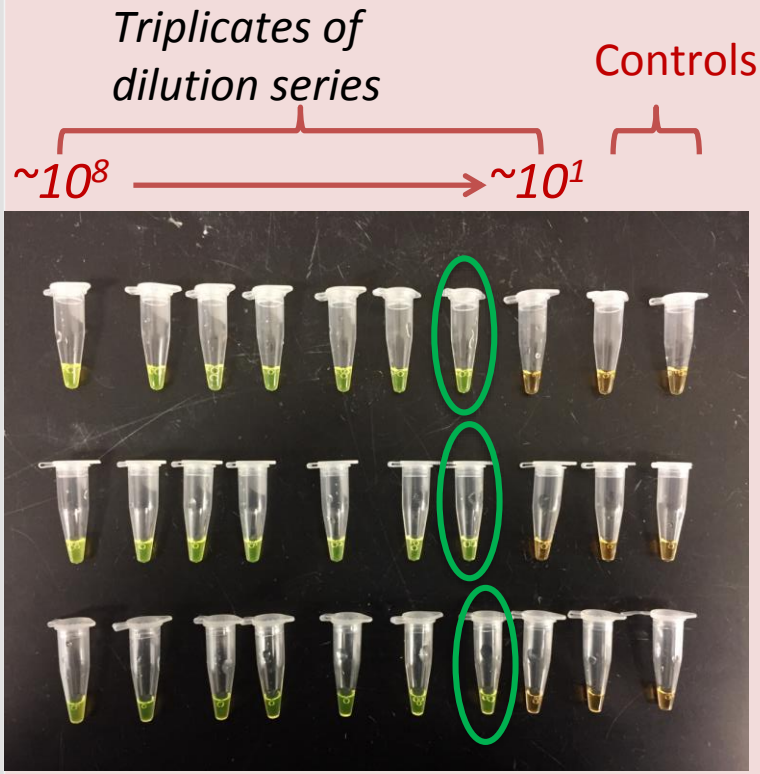
Dilution series 3

Alternate Platforms for Detection

Detection Limits with Direct Amplification

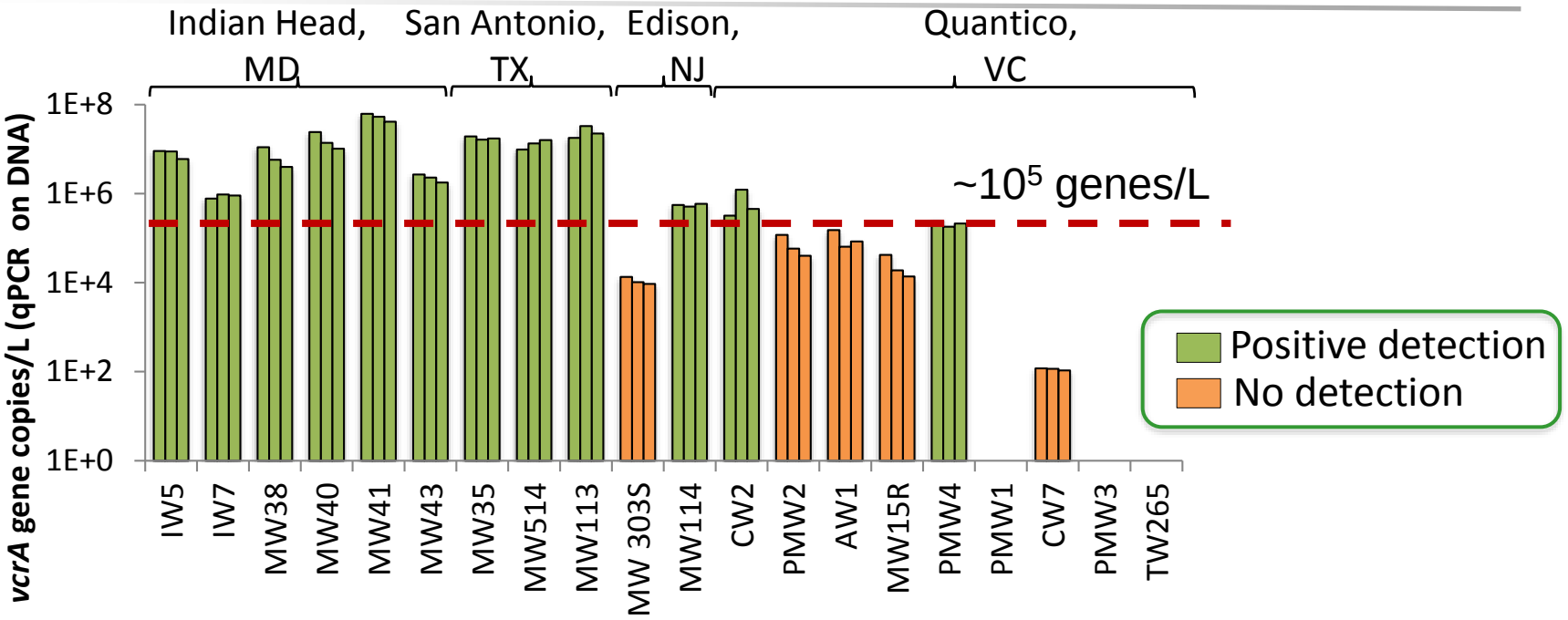
- Detection limits of the SYBR Green LAMP assay were evaluated with centrifuged cells prepared from diluted SDC-9 culture
- An eight fold 10X dilution series was containing ~8.4 x 10^7 gene copies to ~8 gene copies were used as templates
- SYBR Green LAMP assay was able to detect ~84 *vcrA* gene copies per reaction when centrifuged cells were used as a templates for amplification
- Similar results were observed for the *tceA* gene

Detection limits with centrifuged cell templates (vcrA gene)



Alternate Platforms for Detection

Visual Detection of Centrifuged Cell Templates (Groundwater)



- SYBR Green LAMP assay were evaluated with centrifuged cells prepared from groundwater samples from 4 different sites.
- LAMP assay detected *vcrA* gene above ~10^5 genes/L
- >10^6 gene copies/L required for efficient dechlorination*

If all three replicates remain orange, bioaugmentation or biostimulation may be necessary

*Lebrón, C. A., E. Petrovskis, F. Löffler & K. Henn, Jan 2011, Guidance Protocol, ER-0518

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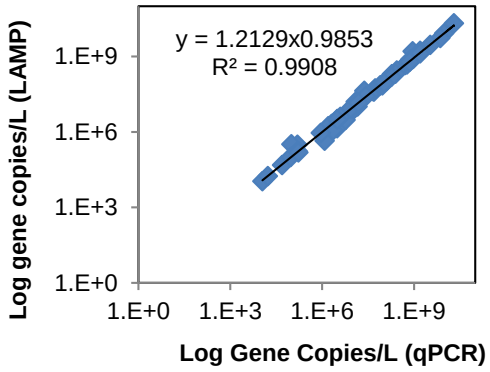
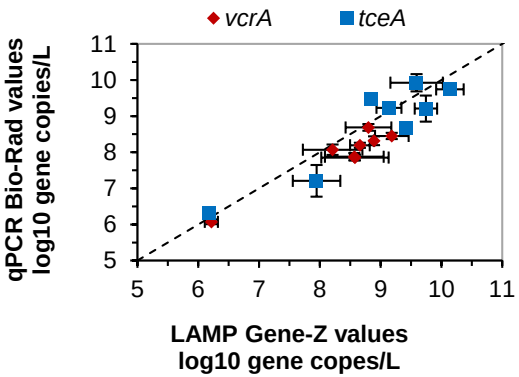
Conclusions

Lessons Learned and Current Research

- LAMP and qPCR produced similar results when DNA templates were used.
- This suggests that LAMP can be used to track the growth of *Dehalococcoides* spp. in cultures and groundwater



- LAMP in Gene Z produced similar results as qPCR in a thermal cycler
- This suggests that LAMP can be performed on alternative platforms and may not necessarily require real time thermal cyclers



- Visual based LAMP assay has the potential for field deployment

- Current research focuses on making the LAMP based assay quantitative (MPN)

Acknowledgements

Funding:

Strategic Environmental Research and Development Program Project ER- 2309 (Contract W912HQ-13-C-0071)

I would like to thank

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- **Phil Dennis from SiREM** for supplying KB-1 culture
- **Frank Löffler from University of Tennessee at Knoxville** for providing the *tceA* plasmid standard