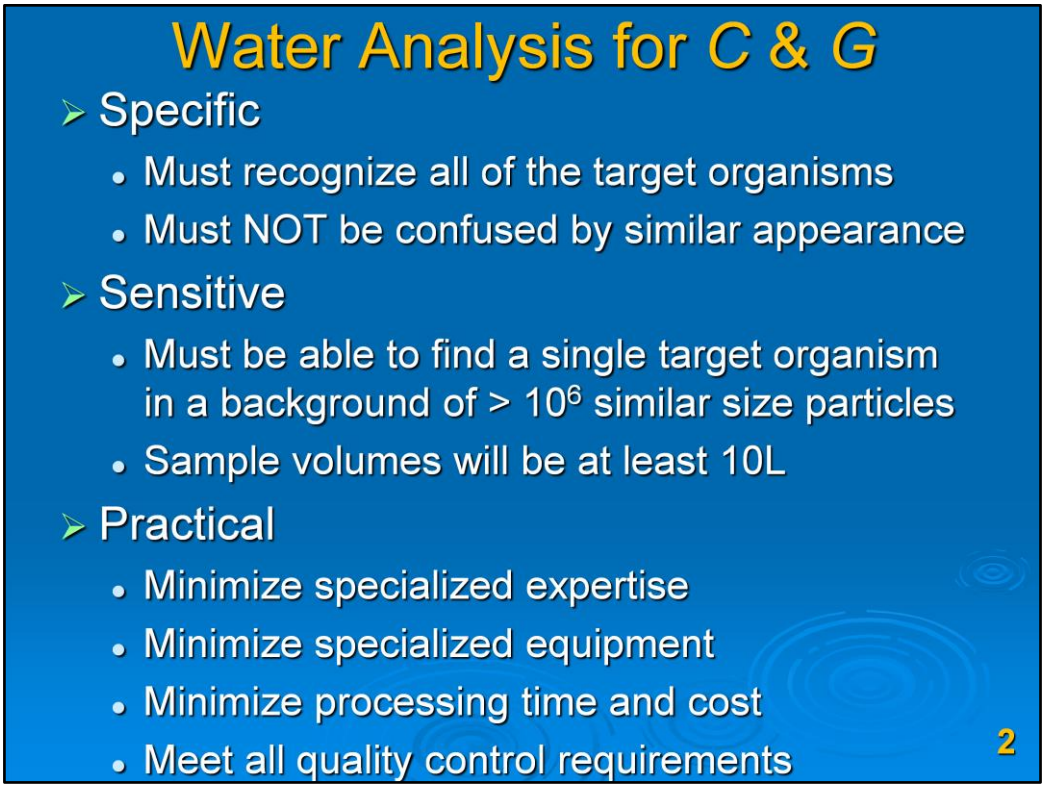
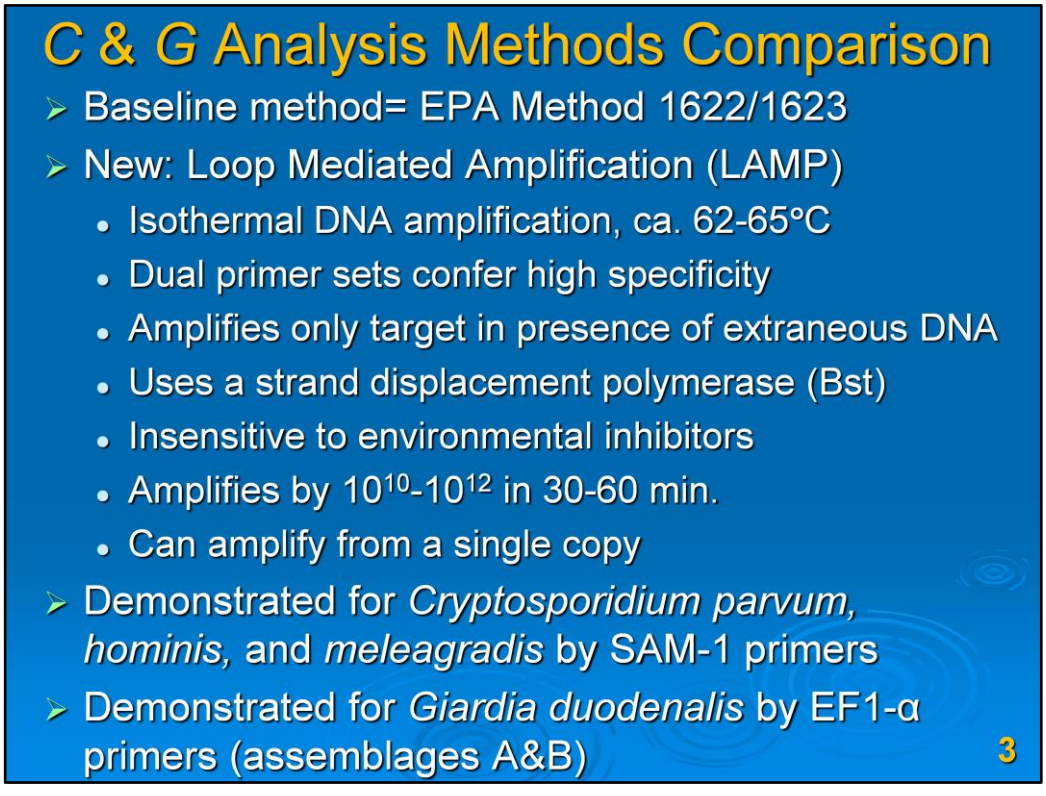
This work was the doctoral project of Frhat M. A. Saaed, PhD, conducted at the University of Wollongong in 2008-2011. Briefly, a LAMP procedure had been previously demonstrated for both *Giardia* and *Cryptosporidium* in surface water samples. This project was to adapt the LAMP procedure to both **simultaneous** and **quantitative** application…and to compare performance of the LAMP-based procedure to that of EPA Method 1623.



Key features of an analytical procedure applied as criteria in this project are:

1. The procedure must produce reliably **SPECIFIC** results finding all but only the target organisms…*Cryptosporidium* oocysts and *Giardia* cysts.
2. The procedure must be **SENSITIVE**…able to find the target organism(s) in samples of at least 10 L in a background of $>10^6$ particles of similar size including both organism and debris.
3. The procedure must be **PRACTICAL**…requiring a minimum of :
    1. Specialized expertise;
    2. Specialized and expensive equipment;
    3. Processing time and expense; and
    4. Meet rigorous quality control requirements



The basis for comparison in this project was EPA Method 1623

Here, LAMP was used for detection adapted to processing required for monitoring *Crypto* & *Giardia* in surface water.

LAMP is a nucleic acid (DNA) amplification procedure using:

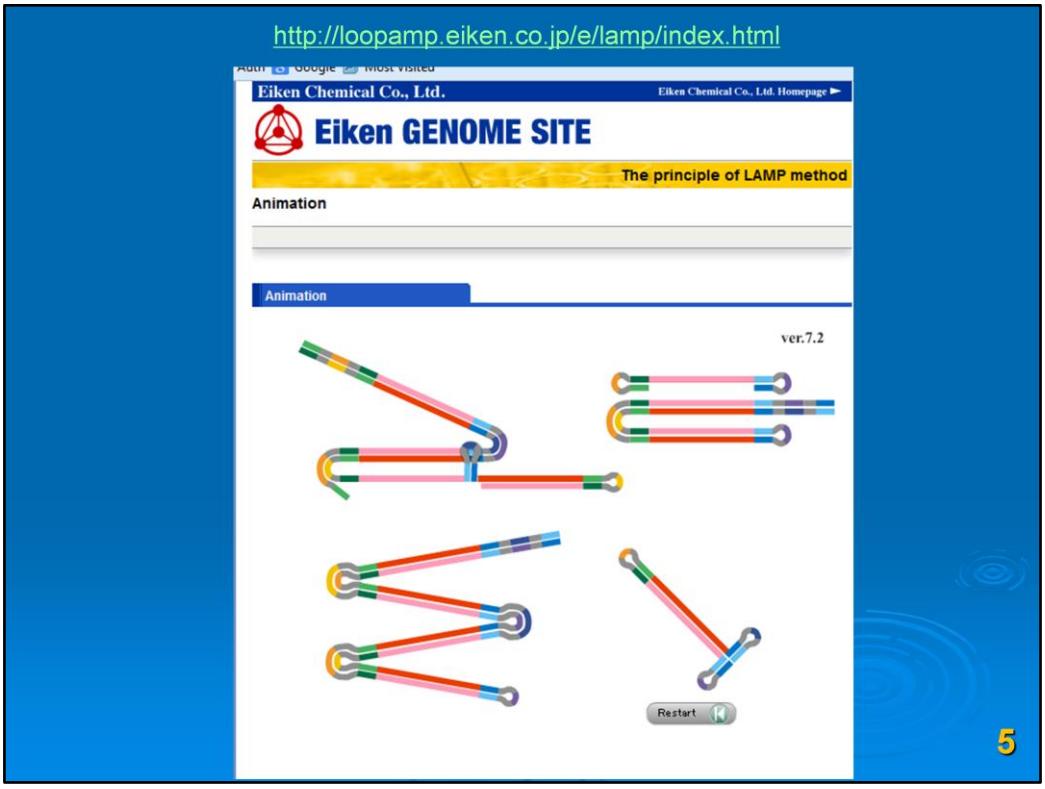
1. Isothermal incubation at ca. 62-65°C
2. Two primer sets allow recognizing only **target** DNA amid DNA from extraneous, even closely related organisms
3. A robust strand-displacement polymerase (Bst)

These features make the procedure:

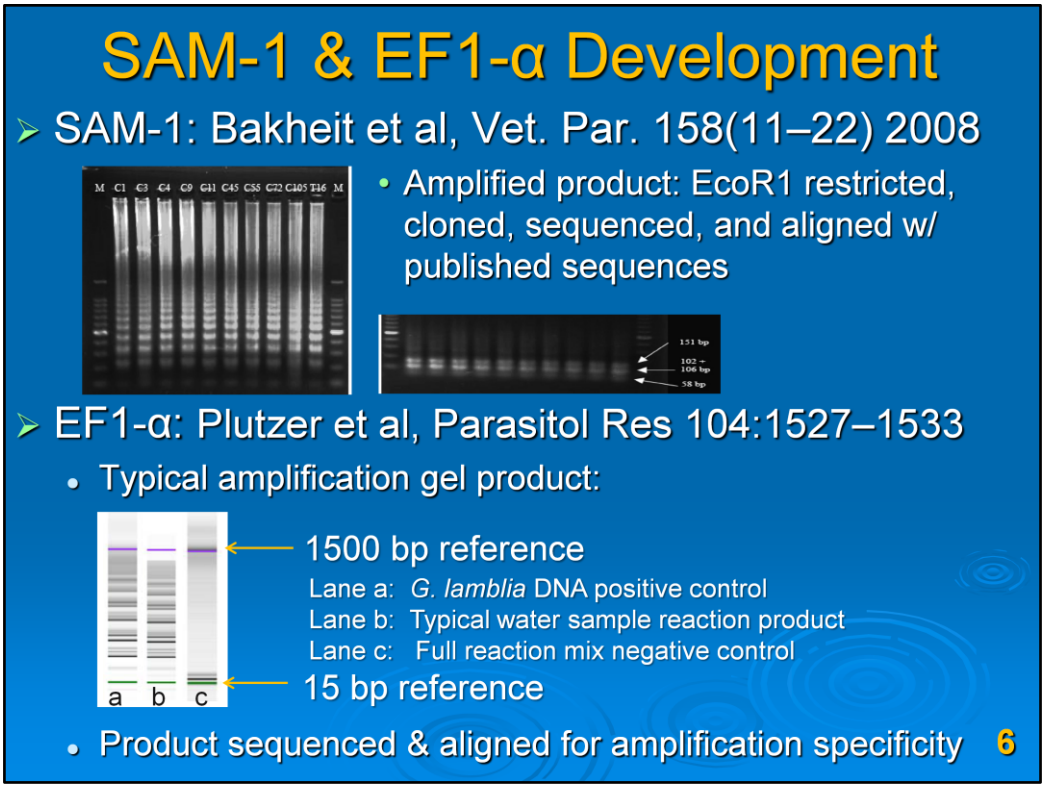
1. Insensitive to environmental interferences
2. Ability to amplify by 10^{10} to 10^{12} in 30-60 min
3. Ability to amplify from a single DNA copy

LAMP is previously demonstrated previously for: 1) *C* parvum, hominis, & meleagradis by SAM-1 gene primers; and 2) *G* doudenalis (assemblages A & B) by EF1-α primers.

An animated description of primer and polymerase actions to amplify DNA using LAMP is available at the Eiken Chemical Co. website.

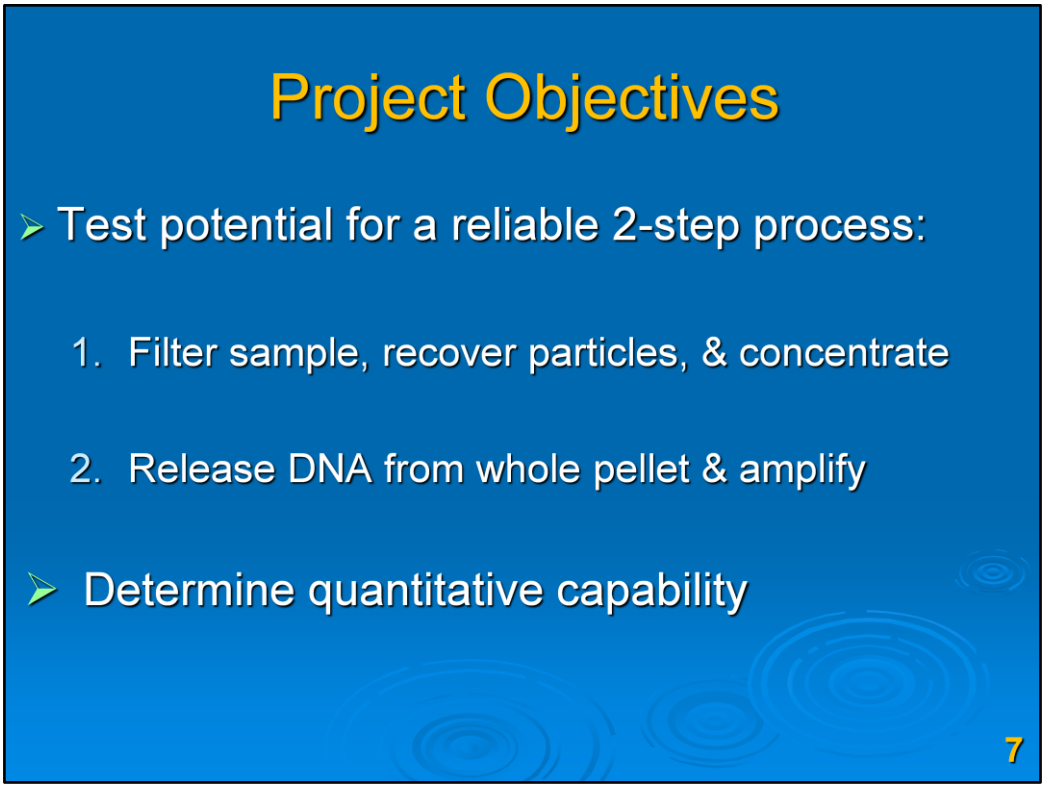


The LAMP process results in progressive multiples of the basic amplification unit identified by the primers in a stem-loop "cauliflower-like" pattern. This results in a characteristic ladder banding pattern when the DNA product is electrophoresed as shown in the next slide.

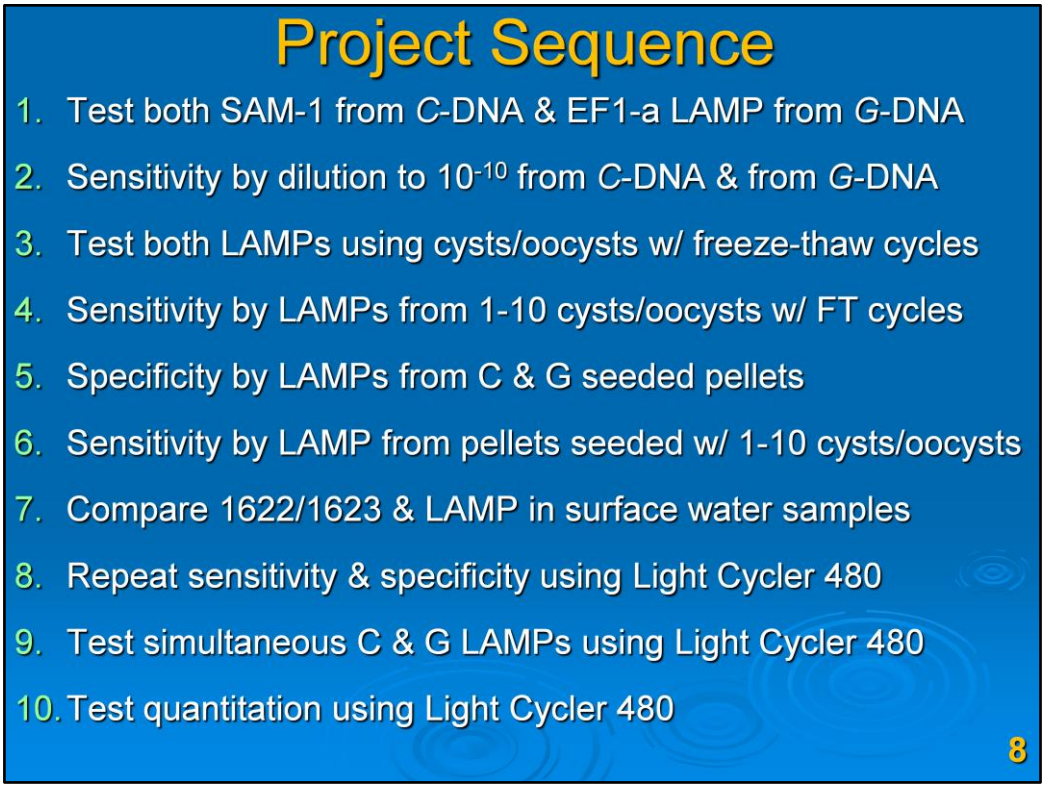


Examples of the SAM-1 gene primer application for detection of *Cryptosporidium* in surface water (Bakheit et al, 2008) and of the EF1-α gene primers for detection of *Giardia* in water and wastewater samples (Plutzer et al, 2009) show the characteristic ladder banding pattern.

Important to the current project: 1) Dr. Ongerth participated in the previous work; 2) the primers described were used in this project using identical reaction mix and application conditions; and 3) amplified product in the previous projects were sequenced and aligned with published sequences to demonstrate the specificity of the amplified product



This project was designed to explore the potential for simplifying the process of monitoring for *Cryptosporidium* and *Giardia* in water.



The project followed a step-by-step process to establish LAMP assay capabilities: 1) Repeat published amplification of *Crypto* & *Giardia* **DNA**: 2) Detect DNA directly from **oocysts** and **cysts** using freeze thaw cycles; 3) Robustness of reaction components **after FT cycles**; 4) LAMP sensitivity (a) by DNA dilution, (b) by small numbers (1-10) of oocysts/cysts; 5) LAMP specificity for C&G seeded to crude pellets from local surface water; 6) finally, simultaneous LAMP for both C & G in pellets w/ extraneous DNA, and explore potential for quantitative RT-LAMP.

Crypto & *Giardia* LAMP from DNA

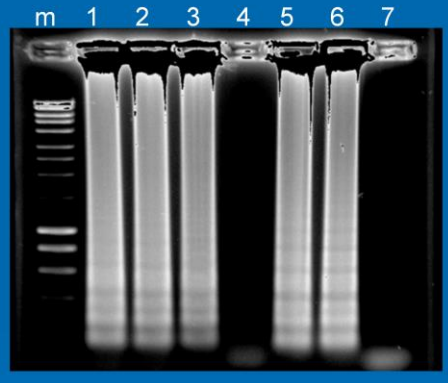


Figure 12. (Lanes 1-3) *Cryptosporidium* DNA LAMP amplification by SAM-1, 63°C for 30 min;(Lane 4) *Crypto* negative control; (Lanes 5-6) *Giardia* DNA by EF1-α, 63°C for 60 min; (Lane 7) *Giardia* negative control

9

After initial optimization, application of the SAM-1 LAMP to DNA from 3 different *Cryptosporidium* sources and application of the EF1-α LAMP to DNA from 2 different *Giardia* sources showed that amplification was successful and that negative controls were clean.

Cryptosporidium SAM-1 LAMP Sensitivity

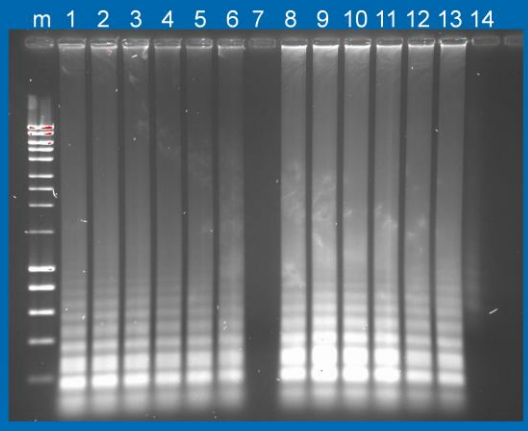


Figure 13. LAMP amplification of *Cryptosporidium* C-1 and 7054 DNA diluted from 10^{-1} to 10^{-10} using SAM-1 primers at 63°C for 30 minutes. Negative controls, (Lanes 7 & 14) Full *Cryptosporidium* reaction mix reagent blank.

10

Sensitivity testing of the SAM-1 LAMP showed ability to amplify from DNA equivalent to less than 1 oocyst…using DNA isolated from 2 independent bovine calf sources of *Cryptosporidium*

Giardia EF1-α LAMP Sensitivity

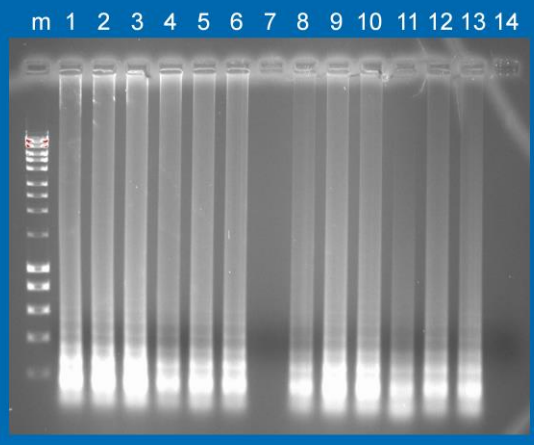
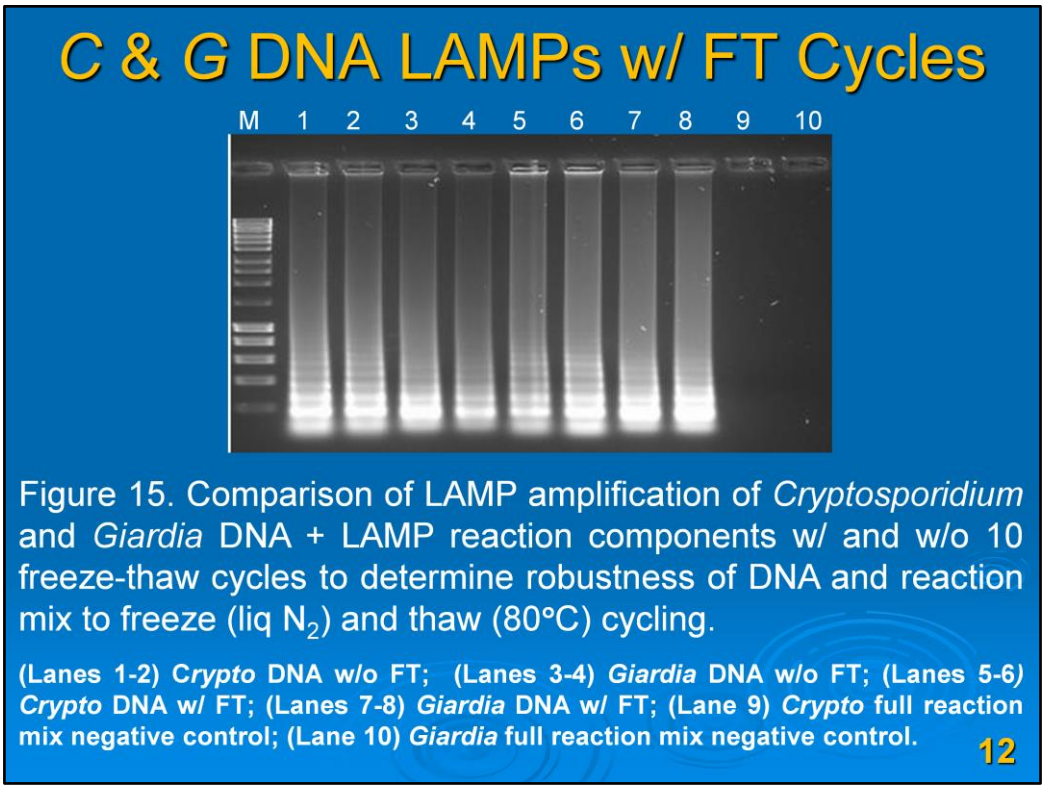


Figure 14. LAMP amplification of *Giardia* G-1 and 7080 DNA diluted from 10^{-1} to 10^{-10} using EF1-α primers at 63°C for 30 minutes. Negative controls (Lanes 7 & 14) full *Giardia* reaction mix reagent blank.

11

Sensitivity testing of the EF1-α LAMP showed ability to amplify from DNA equivalent to less than 1 oocyst…using DNA isolated from 2 independent bovine calf sources of *Giardia.*



Application of both the SAM-1 and EF1a LAMPs to *Cryptosporidium* and to *Giardia* DNA w/ reaction components following exposure to 10 x liq N_2 – 80°C cycles showed that amplification was not impaired.

Oocyst & Cyst LAMPs w/ FT Cycles

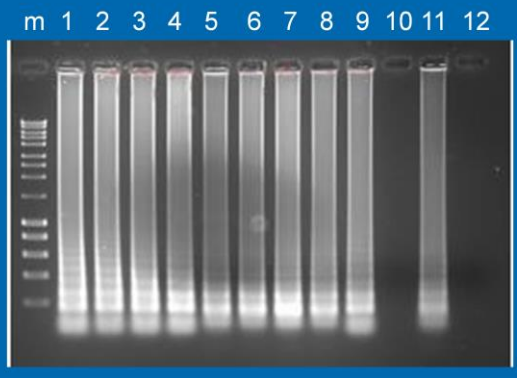


Figure 16. Amplification of *Cryptosporidium* oocyst DNA (lanes 1-4, duplicate pairs of 10 & 100 oocysts) and *Giardia* cyst DNA (lanes 5-8, duplicate pairs of 10 & 100 cysts) directly by 10 freeze-thaw cycles, with positive DNA (lanes 9 and 11) and negative full reaction mix (lanes 10 & 12) controls.

13

Next, replicates of 10 & 100 oocysts and cysts in reaction mix were exposed to FT cycles, then amplified showing that the FT process made the DNA available for amplification

Oocyst LAMP--Low Numbers w/ FT

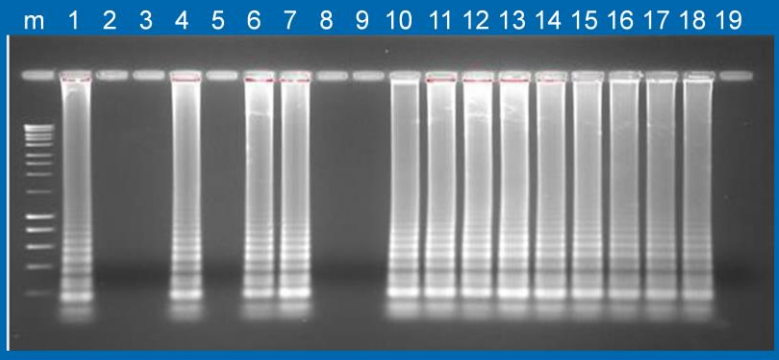


Figure 17. SAM-1 LAMP amplification of *Cryptosporidium* DNA from oocysts w/ 10 freeze-thaw cycles: (lanes 1-5) 1 oocyst; (lanes 6-10) 2 oocysts; (lanes 11-14) 3 oocysts; and (lanes 15-17) 4 oocysts; (lane 18) C 7056 DNA positive; (lane 19) full *Crypto* reaction mix negative control

14

Repeating the previous test using small numbers (1, 2, 3, & 4) of Cryptosporidium oocysts demonstrated the effectiveness of the FT process and the sensitivity of SAM-1 LAMP amplifications.

(*<u>Note</u>: Our interpretation of negative results for 3 of 5 single oocyst and for 2 of 5 two oocyst amplifications indicated that some of the organisms were empty shells, likely due to storage of the oocysts for several months prior to this experiment.)*

Cyst LAMP-- Low Numbers w/ FT

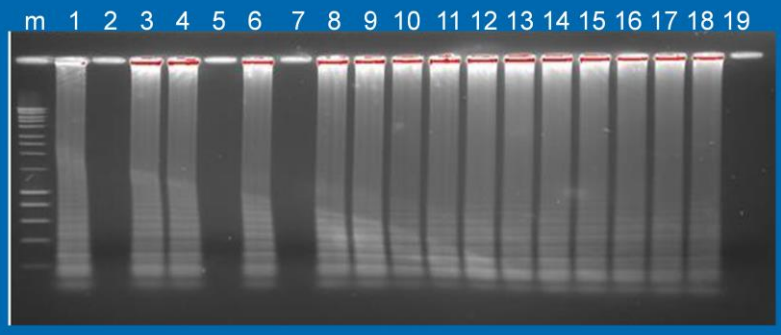


Figure 18. EF1-α LAMP amplification of *Giardia* DNA from cysts w/ 10 freeze-thaw cycles: (lanes 1-5) 1 cyst; (lanes 6-10) 2 cysts; (lanes11-14) 3 cysts; (lanes 15-17) 4 cysts: (lane 18) G 7080 DNA positive control; (lane 19) full EF1-α reaction mix negative control.

15

Similar to the previous test using small numbers (1, 2, 3, & 4) of *Giardia* cysts again demonstrated the effectiveness of the FT process and the sensitivity of the EF1-α LAMP amplifications.

(Note: As for the previous slide, negatives from 1's and 2's were likely empty cysts.)

LAMP Specificity--Pellets w/o *Crypto*

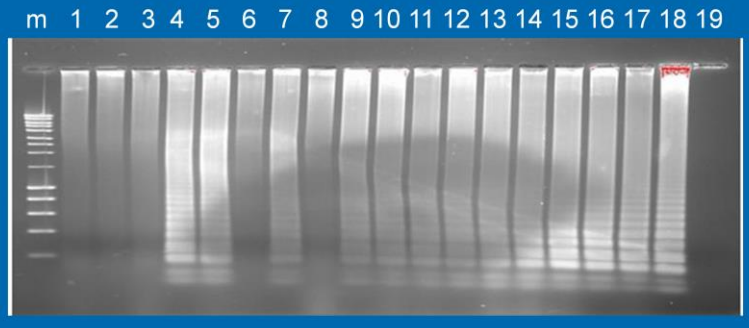


Figure 19. SAM-1 LAMP amplification of *Cryptosporidium* DNA from oocysts added to IMS-cleaned surface water sample pellets: (lanes 1-5) 1 oocyst, (lanes 6-10) 2 oocysts, (lanes 11-14) 3 oocysts; (lanes 15-17) 4 oocysts; (lane 18) C-7056 DNA positive control; (lane 19) full SAM-1 reaction mix negative control.

16

Here, small numbers (1-4) of oocysts were added to portions of a pellet isolated from a local surface stream…after the pellet was subject to IMS for removal of any indigenous *Cryptosporidium*. The the results demonstrate the selectivity of the SAM-1 LAMP and lack of interference from environmental factors.

LAMP Specificity--Pellets w/o *Giardia*

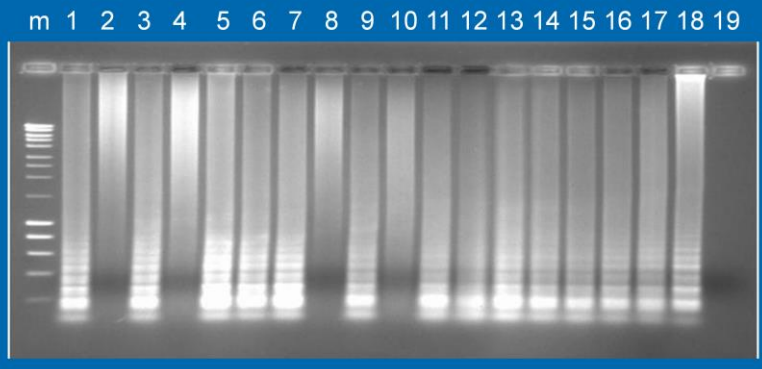


Figure 20. EF1-α LAMP amplification of *Giardia* DNA from addition of cysts to IMS-cleaned surface water sample pellets: (lanes 1-5) 1 cyst; (lanes 6-10) 2 cysts; (lanes 11-14) 3 cysts; (lanes 15-17) 4 cysts; (lane 18) G 7080 DNA positive control, (lane 19) full EF1-α reaction mix negative control .

17

Here, small numbers (1-4) of cysts were added to portions of a pellet isolated from a local surface stream…after the pellet was subject to IMS for removal of any indigenous *Giardia*. The results demonstrate the selectivity of the EF1-α LAMP and lack of interference from environmental factors.

Method1623 *Crypto* & *Giardia* in Ambient Surface Water Sample

Table 14 Analysis of *Cryptosporidium* and *Giardia* by IMS-based procedure for comparison to detection of *Cryptosporidium* and *Giardia* in the pellet by LAMP

Sample No.	Sampling Location	Sample Turbidity NTU	Sample Volume Litre	Crypto. No. In Sample	Giardia, No. In Sample	Crypto. Recovery %	Giardia Recovery %	Crypto Conc. No/ L	Giardia Conc. No./ L
1a	UoW Ck	0.35	10	0	0	30.7	87.0	0.0	<0.11
1b			10	0	0	30.7	87.0	0.0	<0.11
1c			10	0	0	30.7	87.0	0.0	<0.11
1d			10	1	0	30.7	87.0	0.33	<0.11
1e			10	0	0	30.7	87.0	0.0	<0.11
1 Total			50	1	0	30.7	87.0	0.07	<0.023

18

A 50 L sample of surface water processed as 5 x 10 L aliquots was analyzed by EPA Method 1623 for comparison to LAMP assay of a companion 50 L sample taken from the same site at the same time.

Crypto & *Giardia* by LAMP in Ambient Surface Water Sample

Figure 21 Detection of *Cryptosporidium* DNA by SAM-1 LAMP in pellets of 5 x 10 L subsamples of surface water (lanes 1-5) for comparison to IMS-based analysis, Table 14. Lane 6, seeded positive control, Lane 7, MilliQ water negative control.

Fig. 21

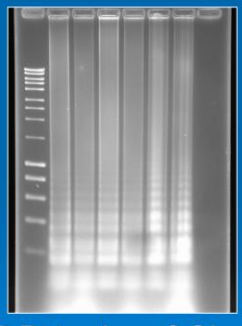


Fig. 22

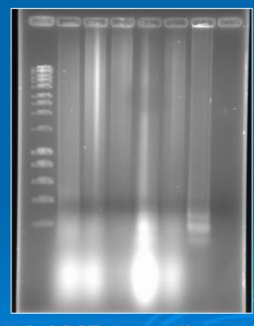


Figure 22 Detection of *Giardia* DNA by SAM-1 LAMP in pellets of 5 x 10 L subsamples of surface water (lanes 1-5) for comparison to IMS-based analysis, Table 14. Lane 6, seeded positive control, Lane 7, MilliQ water negative control.

19

Analysis of the surface water pellets by LAMP showed *Cryptosporidium* presence of in 5 of 5 10 L samples and *Giardia* in 4 of 5 10 L samples indicating the the LAMP assay had greater sensitivity than 1623.

Simultaneous C&G LAMP by Light Cycler 480

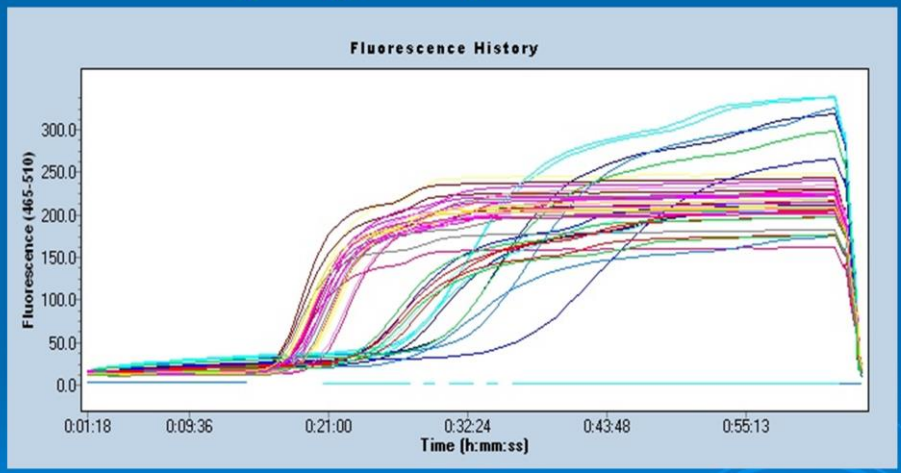


Figure 23 *Cryptosporidium* and *Giardia* DNA Amplification vs. Time using Roche Light Cycler 480

20

Initial testing of amplification using the SAM-1 *Cryptosporidium* LAMP and EF1α *Giardia* LAMP using the Roche Light Cycler 480 produced characteristic RT amplification patterns.

Cryptosporidium RT-LAMP Sensitivity

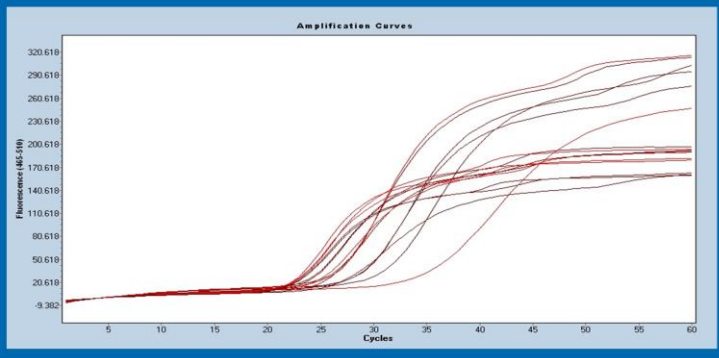


Figure 24. *Cryptosporidium* DNA product (fluorescence) by RT-LAMP using Roche Light Cycler 480 for serially diluted *Cryptosporidium* DNA at concentrations from 10^{-8} to 10^{-12}, (10^{-8}, $10^{-8.5}$, 10^{-9}, $10^{-9.5}$, 10^{-10}, $10^{-10.5}$, 10^{-11}, $10^{-11.5}$, 10^{-12})

21

The RT-LAMP using SAM-1 for *Crypto*. was sufficiently sensitivity to amplify DNA equivalent to less than a single oocyst.

Giardia RT-LAMP Sensitivity

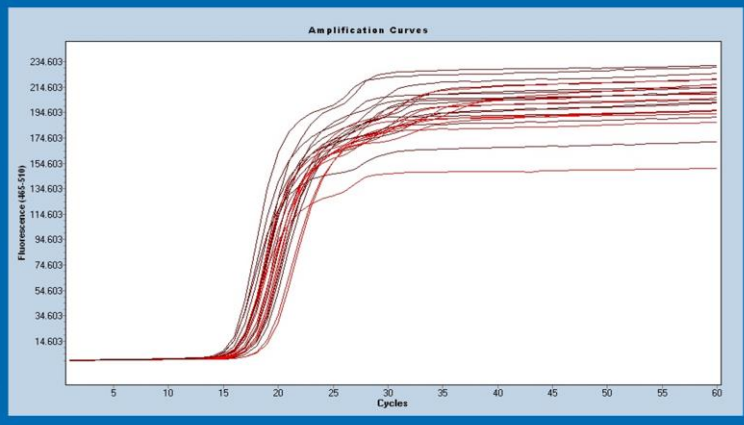
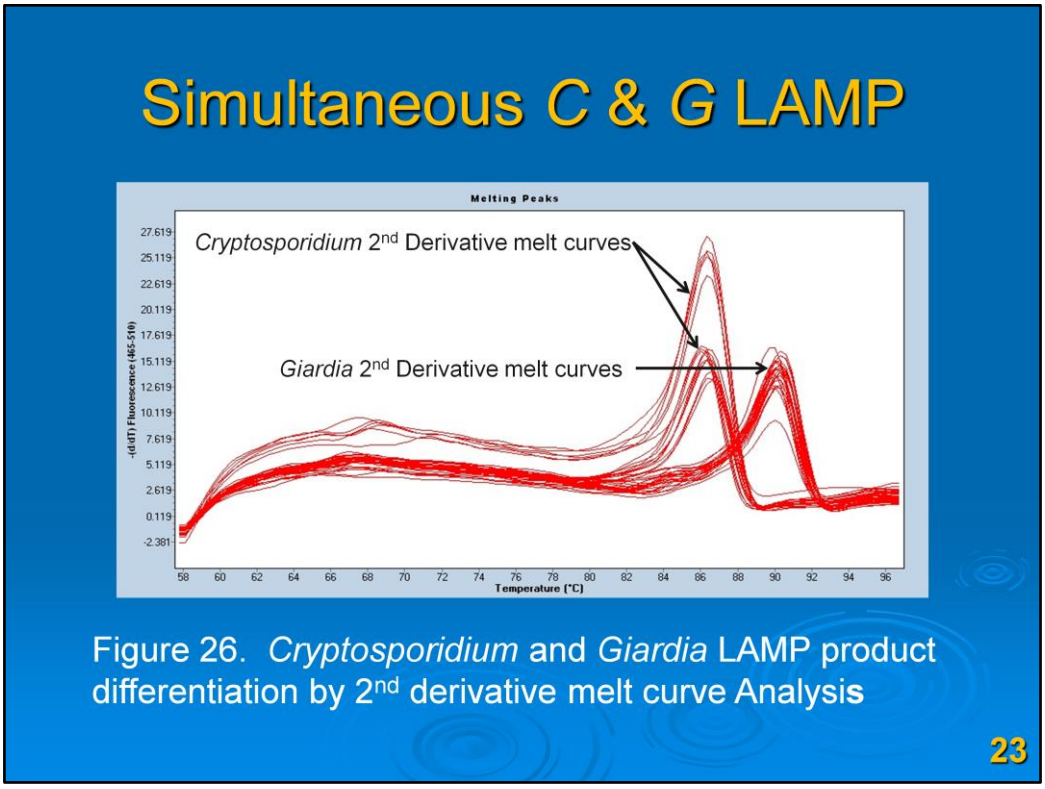


Figure 25 *Giardia* DNA product (fluorescence) by RT-LAMP using Roche Light Cycler 480, for serially diluted *Giardia* at concentrations from 10^{-8} to as little as 10^{-12} , (10^{-8}, $10^{-8.5}$, 10^{-9}, $10^{-9.5}$, 10^{-10}, $10^{-10.5}$, 10^{-11}, $10^{-11.5}$, 10^{-12}).

22

The RT-LAMP using EF1-α for *Giardia* was sufficiently sensitivity to amplify DNA equivalent to less than a single cyst.



Combining the SAM-1 and EF1-a primers in single reactions for a range of oocyst and cyst replicates demonstrated that the DNA of both organisms was amplified and that the amplified products could be efficiently distinguished by melt-curve analysis.

Cryptosporidium Quantitation by RT-LAMP

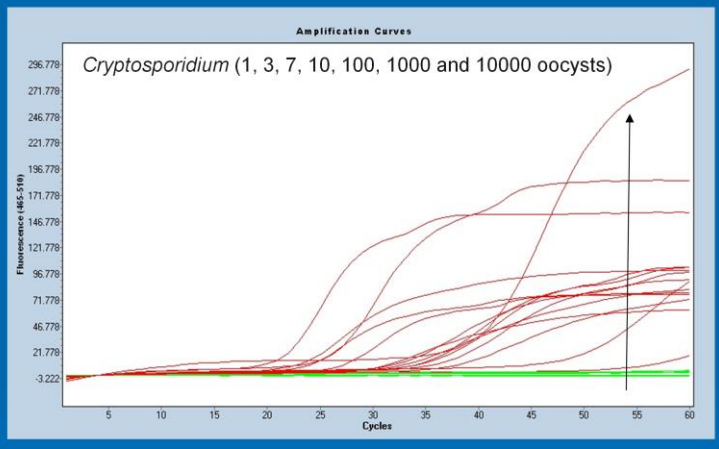


Figure 27. DNA product (fluorescence) from known numbers of *Cryptosporidium* showing the potential for quantitation for small organisms numbers (1, 3, 7, 10, 100, 1000 and 10000) using Light Cycler 480 RT-LAMP.

24

Testing of ability to quantify integer numbers of *Cryptosporidium* oocysts using the SAM-1 by RT-LAMP produced amplified product but results inconsistent with the numbers of oocysts used. This suggests variation in the DNA/oocyst, likely due to relatively long storage of the oocysts.

Giardia Quantitation by RT-LAMP

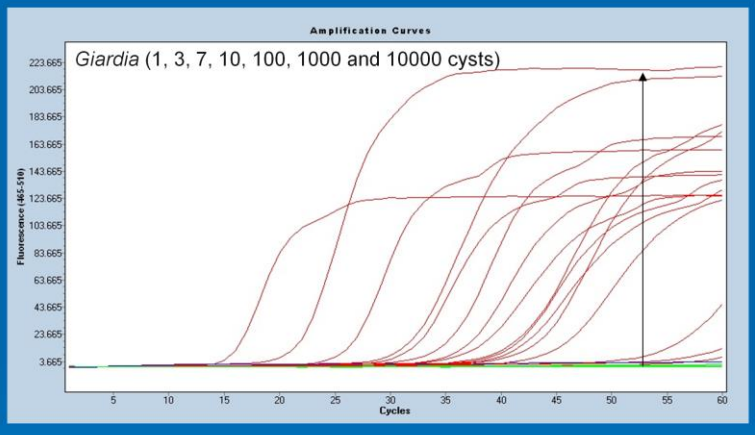


Figure 28. DNA product (fluorescence) from known numbers of *Giardia* showing the potential for quantitation for small numbers of organisms (1, 3, 7, 10, 100, 1000 and 10000) using Light Cycler 480 RT-LAMP

25

Testing of ability to quantify integer numbers of *Giardia cysts* using the SAM-1 by RT-LAMP produced amplified product but results inconsistent with the numbers of cysts used. This suggests likely variation in the DNA/cyst, likely due to relatively long storage of the cysts.

SUMMARY

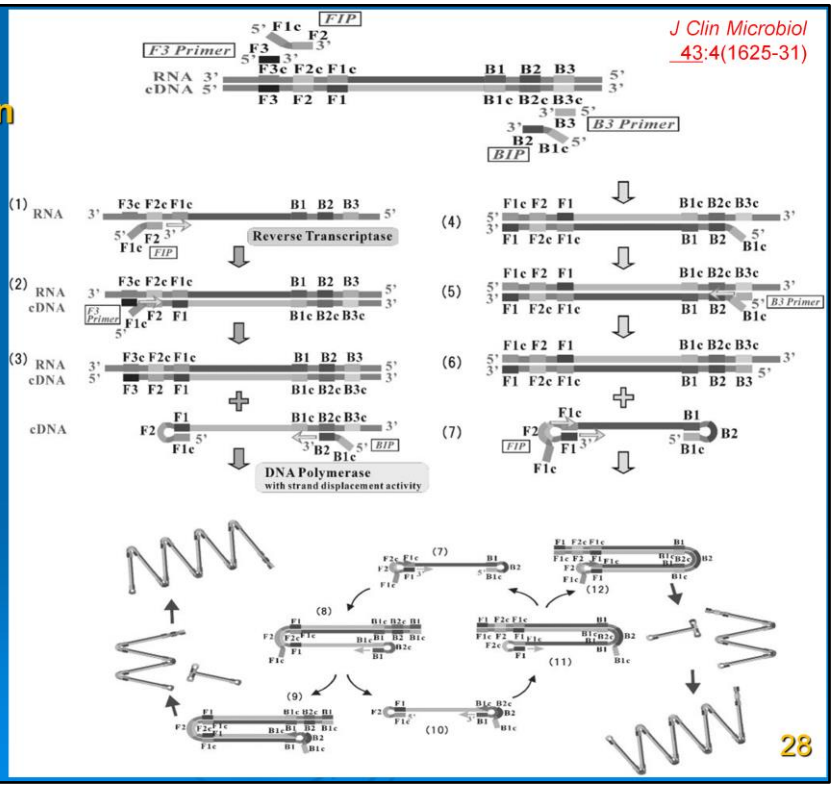
- A simplified LAMP-based 2-step analysis procedure for *Cryptosporidium* and *Giardia* in water is technically feasible
- Quantitative *Cryptosporidium* and *Giardia* analysis in water samples using RT-LAMP analysis appears possible
- Further work to resolve quantitation of organism DNA variation will be useful

26

QUESTIONS ???

27

LAMP Amplification Schematic Diagram



J Clin Microbiol 43:4(1625-31)

28