Total-Fix® Verification Studies

Objective 2 – Compare PCR Performance of Total-Fix® to Common Preservatives/Fixatives over Time

Proposal #: MCC-001
Proposal Date: October 8th, 2012
Report Date: April 4th, 2012

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A. Introduction

The goal of this objective was to compare the PCR performance of stool samples preserved in Total-Fix® versus 8 other commonly-used preservatives or storage methods. The DNA extraction protocol for NucliSENS® easyMag® established in Objective 1 was used in this evaluation.

B. Methods

Sample Preparation
Human stool samples spiked with *Cryptosporidium parvum* and *Giardia lamblia* were pooled, homogenized, and aliquoted into 400 µl amounts to avoid variability from sample to sample and pipetting errors. Each 400 µl stool aliquot containing 10,500 (oo) cysts was preserved in 600 µl of each preservative (Total-Fix®, 10% Formalin, SAF, LV-PVA, Parasafe®, Zinc-PVA, and Ecofix®) at ambient temperature. An additional 400 µl stool aliquot containing 10,500 (oo) cysts was preserved in 600 µl 100% Ethanol and stored at 2-8°C. The control was the 400 µl stool aliquot spiked with10, 500 (oo) cysts in no preservative and stored at -20°C. Time points tested for each condition were storage for 30 minutes, 1 week, 2 weeks, 4 weeks, and 8 weeks.

DNA Extraction
Each preserved sample was extracted simultaneously on the NucliSENS easyMag® using the protocol optimized in **Objective 1**. Preservatives were removed from each sample by centrifugation and then washed with PBS pH 7.2 2 times. Six extractions were completed for each preservative and for each time point.

DNA Amplification
Extracted DNA was amplified and detected using the optimized, multiplex real-time PCR assay, R-Sphere™ CG Assay (in development). Each DNA extract was analyzed by PCR in duplicate.

C. Results

The results for this study are shown in **Tables 1-2** and **Figure 1-2**. Overall, formalin, SAF, and Parasafe® did not perform as well as the other preservatives tested. *C. parvum* and *G. lamblia* DNA extracted from stool preserved in 10% formalin, SAF, and Parasafe® for over 1 week were undetectable by PCR. The preservatives were ranked based upon the PCR performance of both *Cryptosporidium* and *Giardia*. The preservatives that performed best, in order of best to worst performance, include 100% ethanol, Total-Fix®, Eco-fix®, Zinc-PVA, and LV-PVA.

Table 1. PCR Amplification of *Cryptosporidium parvum* by R-Sphere™ CG Assay												
Time Point	**30 minutes**		**Week 1**		**Week 2**		**Week 4**		**Week 8**		**Overall**	
Preservative	*Average*	*Stdev*	*Average*	*Stdev*	*Average*	*Stdev*	*Average*	*Stdev*	*Average*	*Stdev*	*Average*	*Stdev*
Control (No preservative)	33.98	1.30	30.65	0.74	29.54	0.29	29.29	0.34	29.57	1.56	30.61	1.96
100% Ethanol	31.67	0.37	29.76	2.06	28.34	0.19	29.11	0.56	27.60	0.58	29.30	1.55
Total-Fix®	33.21	1.50	32.58	1.02	33.08	1.5	32.00	1.13	30.58	0.44	32.29	1.07
10% Formalin	-	-	-	-	-	-	-	-	-	-	-	-
SAF	-	-	-	-	-	-	-	-	-	-	-	-
LV-PVA	34.27	1.70	37.23	0.84	37.56	1.63	36.37	2.70	34.60	1.89	36.01	1.50
Parasafe®	33.77	0.59	-	-	-	-	-	-	-	-	33.77	-
Zinc-PVA	34.55	2.22	34.52	2.30	35.06	1.27	35.83	1.58	33.06	2.67	34.61	1.01
Ecofix®	34.09	0.68	34.39	0.80	34.76	1.66	34.46	2.31	34.59	2.67	34.46	0.25

Table 2. PCR Amplification of *Giardia lamblia* by R-Sphere™ CG Assay												
Time Point	**30 Minutes**		**Week 1**		**Week 2**		**Week 4**		**Week 8**		**Overall**	
Preservative	*Average*	*Stdev*	*Average*	*Stdev*	*Average*	*Stdev*	*Average*	*Stdev*	*Average*	*Stdev*	*Average*	*Stdev*
Control (No preservative)	28.22	0.42	32.05	0.57	29.37	0.86	29.03	1.01	30.38	2.63	29.81	1.47
100% Ethanol	26.23	0.47	29.06	1.01	27.15	0.76	27.54	0.50	26.74	0.03	27.34	1.08
Total-Fix®	26.87	1.08	30.46	0.80	31.7	1.08	30.34	1.11	31.46	0.56	30.17	1.94
10% Formalin	33.70	2.17	39.04	1.25	-	-	-	-	-	-	36.37	3.77
SAF	34.41	0.42	-	-	-	-	-	-	-	-	34.41	-
LV-PVA	28.33	1.70	31.31	0.67	30.79	0.51	31.56	1.03	31.36	0.56	30.67	1.34
Parasafe®	28.44	2.46	-	-	-	-	-	-	-	-	28.44	-
Zinc-PVA	28.24	0.73	31.48	1.00	30.17	0.69	30.26	0.85	29.93	1.17	30.02	1.16
Ecofix®	28.65	0.29	31.24	0.56	30.04	0.76	29.77	0.64	30.14	1.30	29.97	0.93

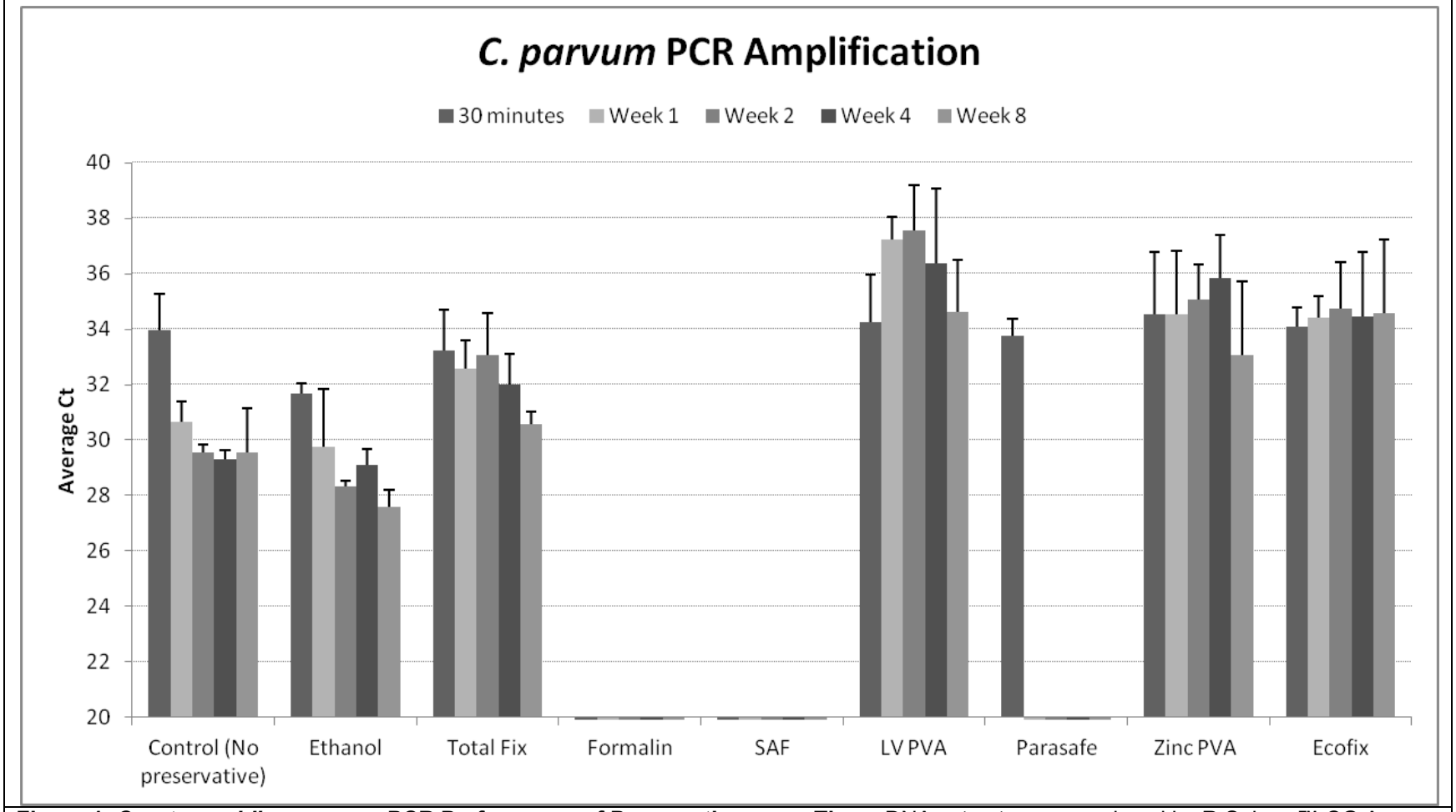

Figure 1. ***Cryptosporidium parvum*** **PCR Performance of Preservatives over Time.** DNA extracts were analyzed by R-Sphere™ CG Assay. Lower Ct values indicate better PCR performance.

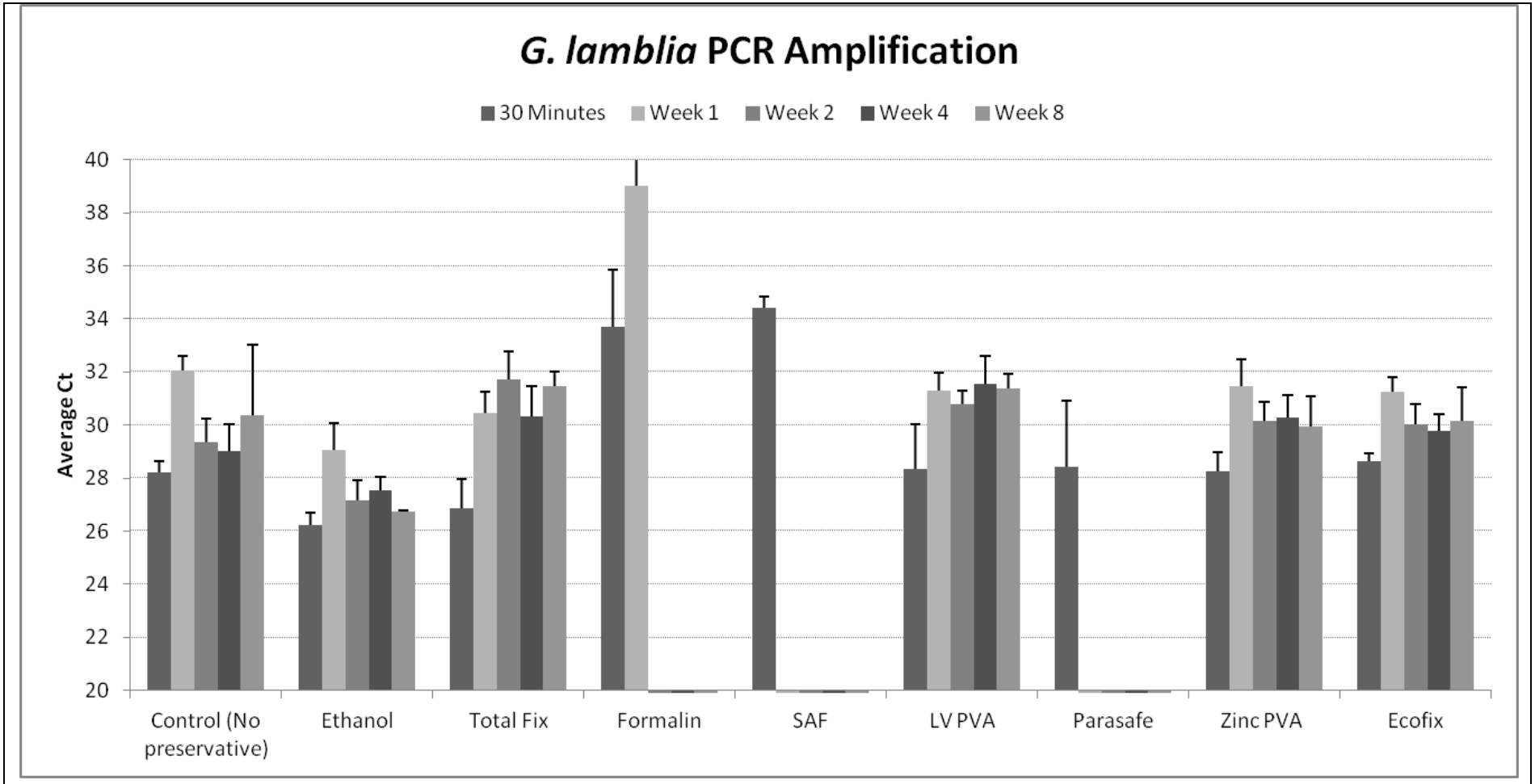

Figure 2. *Giardia lamblia* PCR Performance of Preservatives over Time. DNA extracts were analyzed by R-Sphere™ CG Assay. Lower Ct values indicate better PCR performance.

D. Discussion

Stool samples preserved in 10% formalin, SAF (sodium acetate formalin), and Parasafe® are not suitable for downstream PCR applications. Parasitic DNA extracted from stool in preservatives containing formalin might have been damaged by residual formalin. 100% Ethanol (preserved at 4°C) yields the lowest Ct values for both *Cryptosporidium* and *Giardia* among all other preservatives, including the control (**Table 3**). **Total-Fix® (preserved at room temperature) performs best when compared to Ecofix®, Zinc-PVA, and LV-PVA.**

Table 3. Ct Difference Between Preservative and Control. 10% Formalin, SAF, and Parasafe® are not included due to the lack of any PCR amplification. Negative and lower delta Ct values indicate better PCR performance.

Preservative	***Cryptosporidium***	***Giardia***	**∑Δ Ct**
100% Ethanol	-1.308	-2.466	**-3.774**
Total-Fix®	1.684	0.356	**2.041**
LV-PVA	5.402	0.860	**6.261**
Zinc-PVA	4.000	0.205	**4.205**
Ecofix®	3.853	0.158	**4.011**

Extraction Protocol for Preserved Fecal Specimen for the NucliSENS® easyMAG®

Materials:

Name	Quantity (per Extraction)
1. Preservative Vial	400 µl
2. Fecal Specimen	400 µl
3. PBS pH 7.4	1-3 ml
4. EasyMAG® Lysis Buffer	400 µl
5. EasyMAG® Lysis Buffer 2ml	1 vial
6. EasyMAG® Magnetic Silica	70 µl
7. EasyMAG® Wash Buffer 1, 2, 3	Varied
8. EasyMAG® Disposables	Varied
9. 1.5 - 2.0 ml Microcentrifuge Tubes	1 tube

Equipments:

- ❑ Vortexer
- ❑ Microcentrifuge and bench-top centrifuge
- ❑ Dry bath
- ❑ Biohit pipette and disposable pipette tips
- ❑ NucliSENS® easyMAG® instrument

Things to do before starting:

- ❑ Set up NucliSENS® easyMAG® instrument with the following parameters
  1. Specific A Protocol
  2. Primary Lysis
  3. Feces
  4. Volume: 0.4 mL
  5. Output volume of 70 µ

Procedure:

I. PREPARATION

1. Centrifuge at 14000 x g for 2 minutes. Discard the supernatant.
2. Add **1 ml PBS** into each sample pellet. Vortex until sample is fully homogenized.
3. Centrifuge at 14000 x g for 2 minutes. Discard the supernatant.
4. Add **1 ml PBS** into each sample pellet. Vortex until sample is fully homogenized.
5. Centrifuge at 14000 x g for 2 minutes. Discard the supernatant.

II. LYSIS

6. Add **400 µl easyMAG® Lysis Buffer** to each sample pellet. 1mL of preserved liquid stool has 200 mg of stool.
7. Vortex continuously for 5 minutes.
8. Centrifuge sample at 16000 x g for 2 minutes.
9. Transfer 0.4 ml of supernatant into each sample vessel of the assigned sample strip. Close the instrument lid.
10. Select the Dispense Lysis button and the addition of the lysis buffer will start. The on-board lysis incubation will take approximately 10 minutes to complete.

III. PURIFICATION

11. After the incubation completes, add 70 µl Magnetic Silica into each sample vessel.
12. Mix the sample with using the BioHit pipette (Program 3).
13. Close the instrument lid. Select the Execute Run button to start the run. The run will take approximately 40-45 minutes to complete.
14. After the run ends, transfer extracted DNA from the vessel to new microcentrifuge tubes. Use DNA immediately or store at -20°C for later use.