EDGEWOOD CHEMICAL BIOLOGICAL CENTER
U.S. ARMY RESEARCH, DEVELOPMENT AND ENGINEERING COMMAND
Aberdeen Proving Ground, MD 21010-5424

ECBC-TR-1527

ASSESSMENT OF PAPER TYPES FOR CELL-FREE REACTIONS

Glory Mgboji

OAK RIDGE INSTITUTE FOR SCIENCE AND EDUCATION
Oak Ridge, TN 37830-6209

Steven Blum
Vanessa Funk
Matthew Lux

RESEARCH AND TECHNOLOGY DIRECTORATE

August 2018

Disclaimer

The findings in this report are not to be construed as an official
Department of the Army position unless so designated by other authorizing documents.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
XX-08-2018	Final	Nov 2017–Mar 2018

4. TITLE AND SUBTITLE
Assessment of Paper Types for Cell-Free Reactions

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER
WBS R.0027321.40.1

6. AUTHOR(S)
Mgboji, Glory (ORISE); Blum, Steven; Funk, Vanessa; Lux, Matthew (ECBC)

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Oak Ridge Institute for Science and Education (ORISE); 100 Orau Way, Oak Ridge, TN 37830-6209
Director, ECBC, ATTN: RDCB-DRB-C, APG, MD 21010-5424

8. PERFORMING ORGANIZATION REPORT NUMBER
ECBC-TR-1527

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Office of the Secretary of Defense; 1000 Defense Pentagon, Washington, DC 20301-1400

10. SPONSOR/MONITOR'S ACRONYM(S)
OSD

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release: distribution unlimited.

13. SUPPLEMENTARY NOTES

14. ABSTRACT:
Cell-free systems are currently targeted as a potential method to enhance the detection of chemical and biological threats in the environment. Recent studies have shown a functional way to carry out cell-free reactions on paper. Freeze-drying cell-free reaction solutions onto paper can provide convenient field-forward characteristics such as low weight, easier handling and distribution, and long-term storage capabilities. The work described herein focuses on the exploration and testing of several materials that can be used for cell-free reactions. A variety of chromatography papers and synthetic filter membranes were examined to identify suitable materials for qualities such as portability, stability, flexibility, in-field viability, relatively low cost, and favorable surface properties that allow for quick display and optimum brightness of colorimetric output.

15. SUBJECT TERMS

Cell-free systems	Glass microfiber	β-Galactosidase	Polyethersulfone
Polytetrafluoroethylene	Nylon	Cellulose	Freeze-drying

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
U	U	U	UU	40	Renu B. Rastogi 410-436-7545

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Blank

PREFACE

The work described in this report was authorized under program element number WBS R.0027321.40.1. This work was started in November 2017 and completed in March 2018.

The use of either trade or manufacturers' names in this report does not constitute an official endorsement of any commercial products. This report may not be cited for purposes of advertisement.

This report has been approved for public release.

Acknowledgments

The authors acknowledge the following individuals for their hard work and assistance with the execution of this technical program:

- Applied Research for the Advancement of Science and Technology Priorities, Synthetic Biology for Military Environments collaborators at the Air Force Research Laboratory (Wright-Patterson Air Force Base, OH) and Naval Research Laboratory (Washington, DC) for discussions and related collaborations in developing cell-free systems within the Department of Defense (Arlington, VA); and
- Kelley Betts (U.S. Army Edgewood Chemical Biological Center, Aberdeen Proving Ground, MD) for graphics and editing support.

Blank

CONTENTS

FIGURES

TABLES

Blank

ASSESSMENT OF PAPER TYPES FOR CELL-FREE REACTIONS

1. INTRODUCTION

The relatively recent emergence of synthetic biology has introduced the idea of rewiring and reprogramming existing, well-known systems to achieve novel capabilities and promising advancements in medicine, energy, and defense initiatives.[1] Several useful applications have arisen, from whole-cell biosensors that allow for the detection of chemicals to synthetic probiotics and green energy.[2]

Many of these new developments rely on synthetic gene networks, but significant barriers exist regarding biosafety and host-cell viability outside of controlled environments. Cell-free systems have garnered interest because they provide a means to alleviate some of these concerns.[2] Cell-free systems that contain all of the elements necessary for gene transcription and translation allow for the synthesis of a variety of molecules in contained reactions that are not constrained by the limitations of a living cell.

The U.S. Department of Defense (DoD; Washington, DC) seeks to use such promising technologies to improve and create applications that are designed to aid the Warfighter in the field of duty. In particular, cell-free systems have been targeted as potential methods to enhance the detection of chemical and biological threats in the environment. The DoD is concerned with ensuring that this technology will be appropriate for field use. Recently, Pardee et al. established a functional way to carry out cell-free reactions on paper.[2] Freeze-drying reaction solutions onto paper can provide convenient characteristics, including long-term storage capabilities as well as easier handling and distribution. This document focuses on the exploration and testing of several materials that can be used for cell-free reactions. A variety of chromatography papers and synthetic filter membranes were examined to identify the best options for use with cell-free systems. Suitable materials for our purposes will ideally have the following qualities: portability, stability, flexibility, in-field viability, relatively low cost, and favorable surface properties that allow for the quick display and optimum brightness of colorimetric output.

2. MATERIALS AND METHODS

2.1 Cell-Free Expression

In each experiment, we used the PURExpress cell-free system from New England BioLabs, Inc. (NEB; Ipswich, MA). Kit instructions allowed for the determination of component concentrations. Every 12.5 µL of reaction solution contained plasmid deoxyribonucleic acid (DNA) with a β-galactosidase gene (*lacZ*) insert and chlorophenol red-β-D-galactopyranoside (CPRG) substrate. The typical volumes of the kit components used in a standard 12.5 µL of reaction solution are shown in Table 1. The ribonuclease (RNase) inhibitor used was NEB M0314S at 40,000 units/mL. The *lacZ* gene codes for the hydrolase enzyme LacZ, which catalyzes the hydrolysis of CPRG (a galactoside analog).This cleavage

converts the yellow-orange CPRG substrate into the chromophore chlorophenol red, which results in a dark-purple solution.

Table 1. Standard Volumes of PURExpress *lacZ*/CPRG Reaction Components

Component	Volume (µL)
Solution A (PURExpress)	5.00
Solution B (PURExpress)	3.75
RNAse inhibitor	0.25
diH_2O	1.50
CPRG (12.5 mM)	1.00
lacZ DNA (125 ng/µL)	1.00

diH_2O, deionized water.

To prepare the DNA, the *lacZ* gene was polymerase chain reaction (PCR)-amplified from a plasmid that contained the sequence from *Escherichia coli* BL21. This was accomplished using primers that contained 5′ flanking sequences (shown underlined in this paragraph), which were homologous to the 5′ promoter region and the 3′ terminator region of the pY71 vector (forward: GAGAAATACTAGATGACCATGATTACGGATTCAC; reverse: AGTTATTGTTTATTATTTTTGACACCAGACCAACT).

The 3099 base pair (bp) amplicon was isolated by gel purification (Promega Corporation; Madison, WI), cloned into linearized pY71 vector using the infusion cloning kit (Clontech Laboratories; Palo Alto, CA), and introduced into One-Shot Stellar competent cells (Clontech). Cells were grown, and the DNA was purified using the PureYield Plasmid Midiprep system (Promega) and ethanol precipitation.

2.2 Paper Materials Acquisition and Testing

Five types of chromatography paper and two types of filter paper were acquired from Whatman GE Healthcare Life Sciences (Pittsburgh, PA) for material testing. The characteristics of each type can be found in Table 2.

Table 2. Tested Paper Materials and Respective Characteristics

Product Code	Catalog Number	Characteristics	Price per Ticket*
1 CHR	3001-861	Chromatography paper, 0.18 mm thick, 130 mm/30 min flow rate, largely used for protein and nucleic acid blotting, good for analytical separations	$0.02
2 CHR	3002-917	Chromatography paper, 0.18 mm thick, 115 mm/30 min flow rate, largely used for protein and nucleic acid blotting, recommended for optical/radiometric scanning	$0.02
4 CHR	3004-917	Chromatography paper, 0.21 mm thick, 180 mm/30 min flow rate, largely used for protein and nucleic acid blotting, recommended for routine chromatography with relatively low loading volume	$0.02
20 CHR	3020-917	Chromatography paper, 0.17 mm thick, 85 mm/30 min flow rate, largely used for protein and nucleic acid blotting, recommended for separation of unknown compounds	$0.02
3 MM	3030-861	Chromatography paper, 0.34 mm thick, 130 mm/30 min, industry standard for blotting paper, used largely in electrophoresis procedures	$0.02
42	1442-917	Quantitative filter paper, 0.20 mm thick, 24 mm/30 min flow rate, low ash content, designed for gravimetric analysis and sample preparation for instrumental analysis	$0.03
542	1542-240	Quantitative filter paper, 0.150 mm thick, 60 mm/30 min flow rate, low ash content, acid-hardened, designed for gravimetric analysis and sample preparation for instrumental analysis	$0.08

*The price per ticket was based on the cost of material needed for a standard 24 × 28 mm ticket. It did not include the cost of wax printing, cell-free reaction material, or a housing cartridge.

2.3 Wax Printing and Preparation of Chromatography Paper Matrices

Using a Xerox Phaser 8580 printer (Xerox Corporation; Norwalk, CT), 15-well wax ticket designs were printed onto Whatman chromatography and filter papers. These tickets were then baked at 135 °C for 5 min to allow the wax to permeate the paper material and create wells. After they were baked, each ticket was cut out and blocked in 5% bovine serum albumin (BSA), as described in Pardee et al., for 1 h.[2] The tickets were washed for 20 min in diH_2O and allowed to air-dry.

2.4 Testing Paper Materials

2.4.1 Paper Ticket Setup

Small lip-gloss containers (Qosmedix; Ronkonkoma, NY) were acquired to house each paper ticket during the reaction. To allow these containers to function as hydration chambers, 100 µL of diH_2O was pipetted into the bottom. This would prevent the paper tickets from drying out prematurely. Before a ticket was placed inside the container, the corners of the ticket were cut off to allow for a stable fit. Because the insides of the containers are slightly concave, the tickets were able to be suspended so that the undersides of the wells were not in direct contact with any surface. This positioning allowed the reactions to take place without the components wicking outside of the ticket itself.

2.4.2 Paper Initial Functionality Test (Wet)

An initial functionality test was performed to confirm that the reaction could occur on each type of cellulose-based paper material. Wax printed tickets, made of each of the seven papers, were placed in a container with 100 µL of diH_2O pipetted into the bottom. Then 2.5 µL of PURExpress reaction solution was taken from the standard 12.5 µL reaction tubes and spotted on three wells of each ticket. Negative-control reactions of the same volume, which contained typical reaction components but no DNA, were also spotted on three wells of each ticket. The containers were capped and placed in an incubator at 37 °C. Tickets were visually monitored over time to determine whether a color change occurred. This test was considered to be “wet” because no drying took place between the time of the spotting procedure and when the reaction occurred on the tickets.

2.4.3 Paper Dry Testing

For dry testing, the tickets were spotted with 2.5 µL of PURExpress reaction and negative-control reaction solutions, as described in Section 2.4.2. After spotting, the uncapped containers housing the tickets were placed inside an incubator at 37 °C. When fully dry, the spots on each ticket were rehydrated with 2 µL of diH_2O, and 100 µL of diH_2O was pipetted into the bottom of the container. Containers were capped and placed inside an incubator at 37 °C. Tickets were visually monitored over time to observe occurrence of color change.

2.4.4 Paper Lyophilized Testing

Tickets were spotted with 2.5 µL of PURExpress reaction and negative-control reaction solutions, as described in Section 2.4.2. After spotting, the containers housing the tickets were placed in a –80 °C freezer for 10 min. During this step, containers were capped, but the lids were left untightened. After freezing, the ticket containers were opened and then placed inside a lyophilizer overnight. When the tickets were fully freeze-dried, the spots on each ticket were rehydrated with 2 µL of diH_2O, and 100 µL of diH_2O was pipetted into the bottom of the container. Containers were capped and placed in an incubator at 37 °C. Tickets were visually monitored over time to observe occurrence of color change.

2.4.5 Paper Loading Test

Ideally, the researchers wanted the cell-free reaction to occur within the paper ticket, rather than on top of the surface inside a bead of liquid. To determine the optimal amount of liquid needed to achieve an in-paper reaction, different volumes of CPRG substrate solution were placed on the wells of each type of paper ticket. For paper types 1 CHR, 2 CHR, 4 CHR, 20 CHR, 42, and 542, the volumes of CPRG solution were varied between 0.25 and 2.00 µL, with 0.25 µL intervals. For 3 MM paper, the volumes were varied between 2.00 and 4.50 µL, with 0.50 intervals. The time for the liquid to be completely absorbed into the paper was recorded.

2.4.6 Paper Rehydration Test

To explore how water rehydration volumes affect the speed and intensity of reaction color changes, a rehydration test was designed. For this test, 2.5 µL of PURExpress *lacZ*/CPRG reaction solution was spotted onto each well of each ticket. Tickets were then air-dried in an incubator at 37 °C for 30 min. Various volumes of water were used to rehydrate the wells, as depicted in Figure 1.

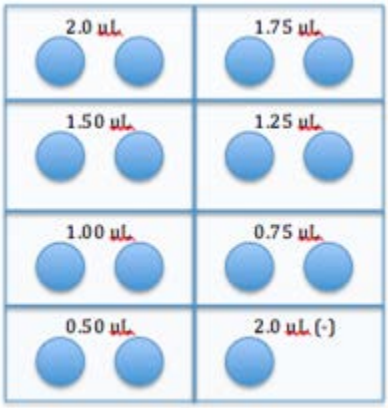


Figure 1. Scheme for rehydration test on paper tickets.

2.5 Acquisition and Testing of Filter Membrane Materials

Several other materials were collected from GE Healthcare Life Sciences, Porex Corporation (Fairburn, GA), and PPG Industries Teslin Substrate Products Business (Monroeville, PA). Their respective sample codes and characteristics are described in Table 3. Most of these nonpaper materials were not able to be wax-printed and were thus tested slightly differently from the chromatography materials, as described later in this document.

Table 3. Synthetic Filter Membrane Materials and Respective Characteristics

Name	Sample Code	Source	Characteristics	Price per Ticket*
Glass microfiber, Grade GF/A	GA	GE 1820-915	0.26 mm thick, features fine retention; suitable for water quality testing and air filtration procedures	$0.12
Glass microfiber, Grade GF/F	GF	GE 1825-915	0.42 mm thick, offers fine particle retention and good loading capacity and flow rate; reliable for critical filtration	$0.45
Glass microfiber, Grade GF/B	GB	GE	0.68 mm thick, has fine particle filter and good loading capacity; useful for liquid clarification	$0.33
Glass microfiber 934-AH	934	GE 1827-866	0.44 mm thick, smooth water quality filters with high retention efficiency; used in total suspended solids analysis	$0.09
Cellulose acetate	OE66	GE 10404139	Low protein binding, hydrophilic	$0.55
Polyethersulfone	PES	GE 111164	Hydrophilic, stable in alkaline pH; suitable for biologic samples and aqueous applications	$0.36
Quartz microfiber	QM	GE 1851-865	0.48 mm thick; used in air sampling filter applications	$0.34
Polytetrafluoroethylene (PTFE)	TE35 or TE	GE 10411413	Chemically stable and inert; withstands aggressive solvents, liquids, and gases	$0.92
PPG Teslin, Grade SP600	SP600 or SP60	Teslin	0.15 mm thick, all-purpose material; recommended for various print technologies, including offset, flexography, gravure, intaglio, thermal transfer, and inkjet	NA
PPG Teslin, Grade DIGITAL1000	DIGI	Teslin	Developed specifically for Xeikon† print models; does not require coating like other synthetics	NA
iSense Semi-sintered PE (Porex affinity filter)	iSen	Porex	1.5 mm thick; designed to capture and purify proteins, peptides, and oligonucleotides	$0.14
Nylon	Nyln	GE 10416094	Moderately charged; designed for southern and northern blotting, as well as plaque lifts and dot-/slot-blotting	$0.76
903 Protein-saver cards (cellulose-based)	Psv	GE	Sample collection cards, 75–80 µL sample on each spot, 3 × 2 in. cards	$0.85

*The price per ticket was based on the cost of material needed for a standard 24 × 28 mm ticket. It did not include the cost of wax printing, cell-free reaction material, or a housing cartridge.
† Xeikon N.V.; Eede, The Netherlands.
PE, polyethylene; NA, not applicable.

Materials mentioned herein, but not described in Table 3, did not advance beyond preliminary testing and did not go on to be considered as suitable materials for the paper-based project.

2.6 Preparation of Nonpaper Filter Membranes

2.6.1 Nonpaper Preliminary Blocking Test

Using GF, GB, OE66, PES, QM, and TE35, which are described in Table 3, preliminary tests were run to determine whether or not any benefits came from blocking the filter membranes with 5% Tween 20.[2] Each filter membrane was made into 6 mm circular cutouts because they could not be readily wax-printed like the paper materials. To block the filter membranes, the circle cutouts were placed in microcentrifuge tubes with 1 mL of 5% Tween 20 and rotated for 1 h. They were then placed in microcentrifuge tubes with diH_2O for a 20 min rotating wash. After the materials were air-dried, they were ready for the blocking test.

Blocked and unblocked cutouts of the six filter membranes were suspended in 1.5 mL microcentrifuge tubes with 100 µL of water in the bottom. Two pipette tips were cut to suspend the membranes within the microcentrifuge tube and to keep them in place. PURExpress *lacZ*/CPRG reaction solutions were created, and 2.5 µL of reaction solution was placed at the center of each circular filter membrane. The tubes were then capped and monitored to determine occurrence of color change.

2.6.2 Preliminary Filter Membrane and Synthetic Paper Comparison Test

Teslin materials with the following sample codes were prepared in a manner exactly as described in Section 2.6.1 for the unblocked cutouts: SP600, SP700, SP800, SP1000, SP1200, SP1400, HD1400, BLUE1000, DIGITAL1000, SPID1000, TS1000, and IJWP1000. A circular cutout of iSense PE (sample code: iSen) was prepared and tested as in Section 2.6.1 with the Teslin materials. The reaction was monitored to see whether a color change from yellow to purple took place.

2.6.3 Nonpaper Air-Dried Reaction Test

Materials with the following sample codes were used for the air-dried reaction test: QM, GF, GB, TE, SP600, SP800, SP700, and iSen. Unblocked 6 mm discs of each material were cut out and placed in 1.5 mL microcentrifuge tubes. Then 100 µL of diH_2O was placed in the bottom of each tube to keep the material hydrated. Two pipette tips were cut to suspend the membrane and keep it in place. A PURExpress reaction master mix containing *lacZ* and CPRG at their typical concentrations (Section 2.1) was created, and 2.5 µL of the reaction solution was placed at the center of each membrane. The tubes were left uncapped and placed in an incubator at 37 °C to be air-dried. The membranes were then rehydrated by spotting 2.5 µL of diH_2O on their centers. After this, the tubes were capped and placed back into the incubator at 37 °C to be monitored for color changes.

2.6.4 Nonpaper Air-Dried Reaction Test: Increased Reaction and Rehydration Volume

Materials with the following sample codes were used for this modified air-dried reaction test: QM, GB, GF, and 3MM. One material, 3MM, was used as a positive control because previous experiments showed that it would produce a color change after complete absorption of the reaction solution. The 6 mm discs, reaction tubes, and *lacZ*/CPRG reaction solutions were prepared in accordance with the original air-dried reaction test (Section 2.6.3). This time, however, 5.0 µL of reaction solution was placed at the center of each membrane. The microcentrifuge tubes containing the discs were left uncapped and then placed inside of an incubator at 37 °C to air-dry for 20 min. The tubes were then removed from the incubator, and the membranes were rehydrated by placing 10 µL of diH_2O directly on the centers of the membranes. The tubes were capped and placed in the incubator at 37 °C, where they were monitored for color changes.

2.6.5 Nonpaper Lyophilized Test

Materials with the following sample codes were used for this test: QM, GF, GB, TE, SP600, SP800, SP700, and iSen. The 6 mm discs, reaction tubes, and *lacZ*/CPRG master mixes were prepared in accordance with the original air-dried tests (Section 2.6.3). Then 2.5 µL of reaction solution was placed in the center of each membrane. Capped tubes were placed in a freezer at –80 °C for 10 min, and then the tubes were uncapped and left in a lyophilizer overnight. The membranes with lyophilized reaction solutions were rehydrated by placing 2.5 µL of diH_2O directly on the centers of the membranes. The tubes were then capped and placed in an incubator at 37 °C, where they were monitored for color change.

2.6.6 Transition to 384-Well Plate Setup

After the blocking test, a 384-well plate was used for subsequent testing of the nonpaper filter membranes. For wet, air-dried, and lyophilized tests, 2 mm diameter circles were cut out of each material using a biopsy punch, and the samples were placed in the wells. The *lacZ*/CPRG reaction solutions were then placed directly on the material, and the color change was noted using an HP ScanJet G4050 scanner (Hewlett-Packard; Palo Alto, CA).

2.6.7 Nondried Reaction: Plate Test for Nonpaper and Synthetic Paper

The filter membranes and synthetic materials with the following sample codes were included in the plate: GF, GM, PES, QM, TE35, SP60, DIGI, iSen, GA, 934, and Nyln. The sample codes for the paper materials included in the plate test were PsvU (unblocked), PsvT (blocked 0.05% Tween 20), PsvB (blocked 5% BSA), 42, and 3MM.

Circular pieces of 2.5 mm diameter were cut out of each material with a razor knife. The paper discs (PsvB, 42, and 3MM) were placed in microcentrifuge tubes with 1 mL of 5.0% BSA and rotated for 1 h, and the noncellulose membrane discs were placed in microcentrifuge tubes with 1 mL of 0.05% Tween 20 and rotated for 1 h. The PsvU disc was not blocked. After the blocking phase, all discs were washed with 1.0 mL of water, rotated for

20 min, and then air-dried in a fume hood. Next, the discs were placed in separate wells of a 384-well, clear bottom plate. A master mix of PURExpress *lacZ*/CPRG reaction solution was created using typical concentrations (Section 2.1). A negative-control mix, which did not contain any DNA, was included in the test using one CHR paper. Then 2.0 µL of reaction solution was placed on the center of each membrane. The plate was covered and then placed in an incubator at 37 °C and monitored at 5 min intervals for color changes. Images were taken of the plate using an HP ScanJet G4050 scanner.

2.6.8 Air-Dried Reaction: Plate Test for Nonpaper and Synthetic Paper

Materials with the following sample codes were included in this plate test: GF, GM, PES, QM, iSen, GA, 934, PsvT, PsvB, 42, and 3MM.

Circular shapes of 2.5 mm diameter were cut out of each material with a biopsy punch. The materials were prepared, and the master mix solution was created and spotted as described in the nondried reaction plate test (Section 2.6.7). Upon completion of the spotting, the uncovered plate was placed in an incubator at 37 °C for 15 min. The discs with dried reaction solutions were then rehydrated with 2.0 µL of diH_2O. Next, the plate cover was applied, and the plate was placed in the incubator at 37 °C. It was monitored at 5 min intervals for color change. Images of the plate were taken using an HP ScanJet G4050 scanner.

2.6.9 Lyophilized Reaction: Plate Test for Nonpaper and Synthetic Paper

Materials with the following sample codes were included in this test: GF, GM, PES, QM, iSen, GA, 934, PsvT, PsvB, 42, and 3MM.

Circular shapes of 2.5 mm diameter were cut out of each material with a biopsy punch. The materials were prepared, and the master mix was created and spotted as described in the nondried plate test (Section 2.6.7). Upon completion of the spotting, the plate was covered and placed in a freezer at –80 °C for 15 min. The plate was then placed in a prechilled lyophilizer overnight. The material discs with the freeze-dried reaction solutions were rehydrated with 2.0 µL of diH_2O, and the plate cover was applied. The covered plate was placed in an incubator at –37 °C. The plate was monitored every 5 min for color change. Images were taken using an HP Scanjet G4050 scanner.

3. RESULTS

3.1 Paper Materials Testing Results

3.1.1 Initial Functionality Test Results for Paper

As shown in Figure 2, the *lacZ*/CPRG reaction took place in all five of the chromatography papers and produced a purple color. Thus, all five papers were used in subsequent testing. It should be noted that the 3MM paper exhibited leaking of the reaction solution from the experimental wells to the negative-control wells.

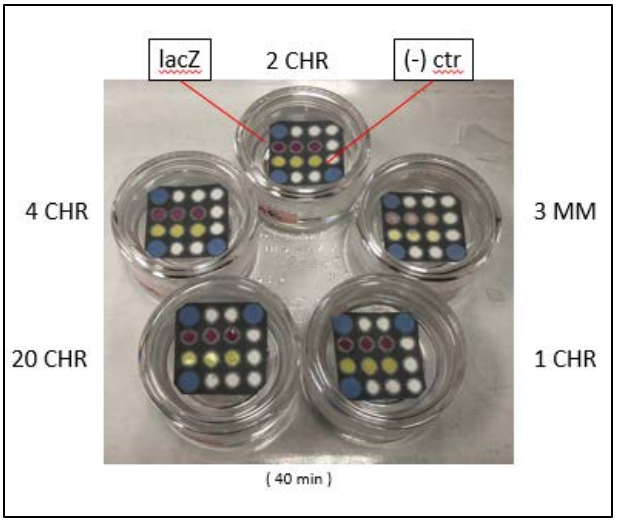


Figure 2. *LacZ*/CPRG reactions. *LacZ*/CPRG reactions produced a purple color on all paper tickets that were wax-printed on 1 CHR, 2 CHR, 4 CHR, 20 CHR, and 3MM paper. 3MM paper exhibited bleed-over from the experimental wells to the negative-control wells.

3.1.2 Dry Test Results for Paper

After the reaction solution was dried onto the tickets and the wells were rehydrated with water, the reaction was able to take place in all tested chromatography papers, as depicted in Figure 3. Again, the 3MM paper exhibited leakage of the reaction solution to other wells on the ticket that were outside of the experimental region. Of the five paper types, 2 CHR exhibited the fastest color change.

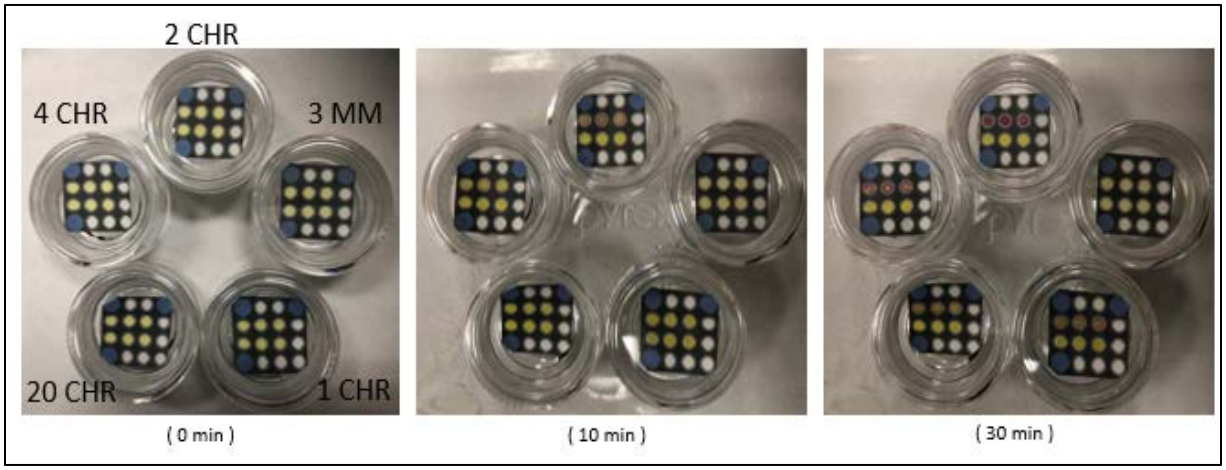


Figure 3. *LacZ*/CPRG reaction progression for dry ticket test. Panels shown on the left, middle, and right show images at times of 0, 10, and 30 min, respectively. The 2 CHR paper displayed the fastest color change.

3.1.3 Lyophilized Test Results for Paper

After the *lacZ*/CPRG reaction solutions were lyophilized on the paper tickets and rehydrated with water, the reaction progressed on all of the chromatography papers except the 3MM, which had wells that remained yellow in color instead of changing to a purple color. The 1 CHR and 20 CHR materials worked better than the other three paper materials in terms of speed of color change and consistency of color across experimental wells.

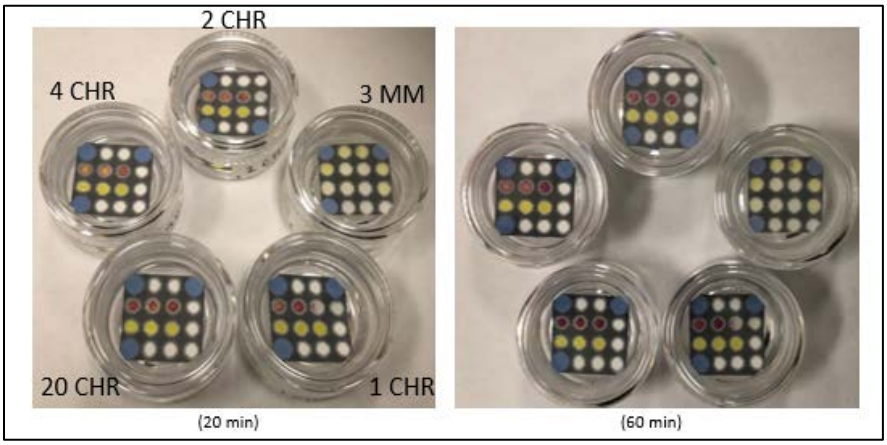


Figure 4. *LacZ*/CPRG reaction progression for lyophilized ticket test. Left and right panels show images at times of 20 and 60 min, respectively.

3.1.4 Loading Test Results for Paper

The time (in seconds) for CPRG solution to be absorbed into each paper material was recorded in Table 4A. The values in green represent the parameters chosen for subsequent testing because they allowed for the highest volume of CPRG liquid to absorb within a reasonable wait time. The thickest of the chromatography papers, 3MM, was the only material to promptly absorb volumes greater than 4 µL in each well. The absorption times for 3MM are shown in Table 4B.

Table 4. Absorption Times

A.

	Paper Type					
Vol. (uL)	1 CHR	2 CHR	4 CHR	20 CHR	Grade 42	Grade 542
2.00	120+	120+	101	120+	120+	120+
1.75	120+	120+	95	120+	120+	120+
1.50	120+	120+	67	120+	120+	120+
1.25	120+	85	7	103	120+	120+
1.00	53	41	instant	37	120+	120+
0.75	7	instant	instant	instant	79	70
0.50	instant	instant	instant	instant	instant	17
0.25	instant	instant	instant	instant	instant	instant

B.

3MM Paper	
Vol. (uL)	Time (s)
2.00	instant
2.50	instant
3.00	5
3.50	4
4.00	8
4.50	45

Notes: (A) Shows the absorption times for various volumes of CPRG solution across multiple paper materials. (B) Shows the absorption times for various volumes of CPRG solution in 3MM paper.

Using the parameters highlighted by the green values in Table 4, the corresponding volumes of *lacZ*/CPRG reaction solution were tried on each paper type. Of the five paper types, 1CHR and 3MM were the only two that produced positive results, as displayed in Figure 5. This result indicates that much of the observed color changes in other testing occurred on the surface of the papers rather than inside the papers.

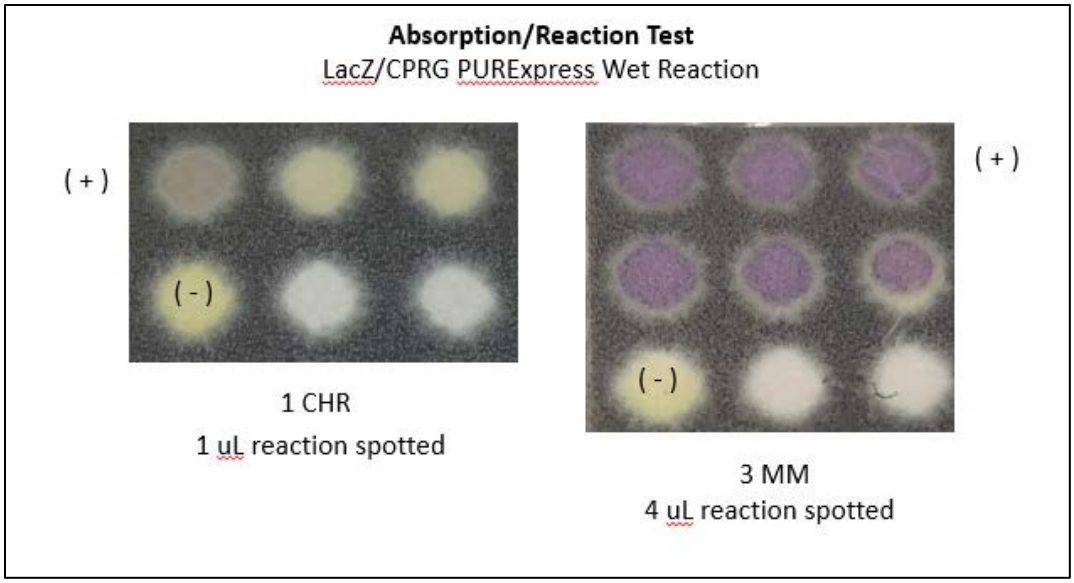
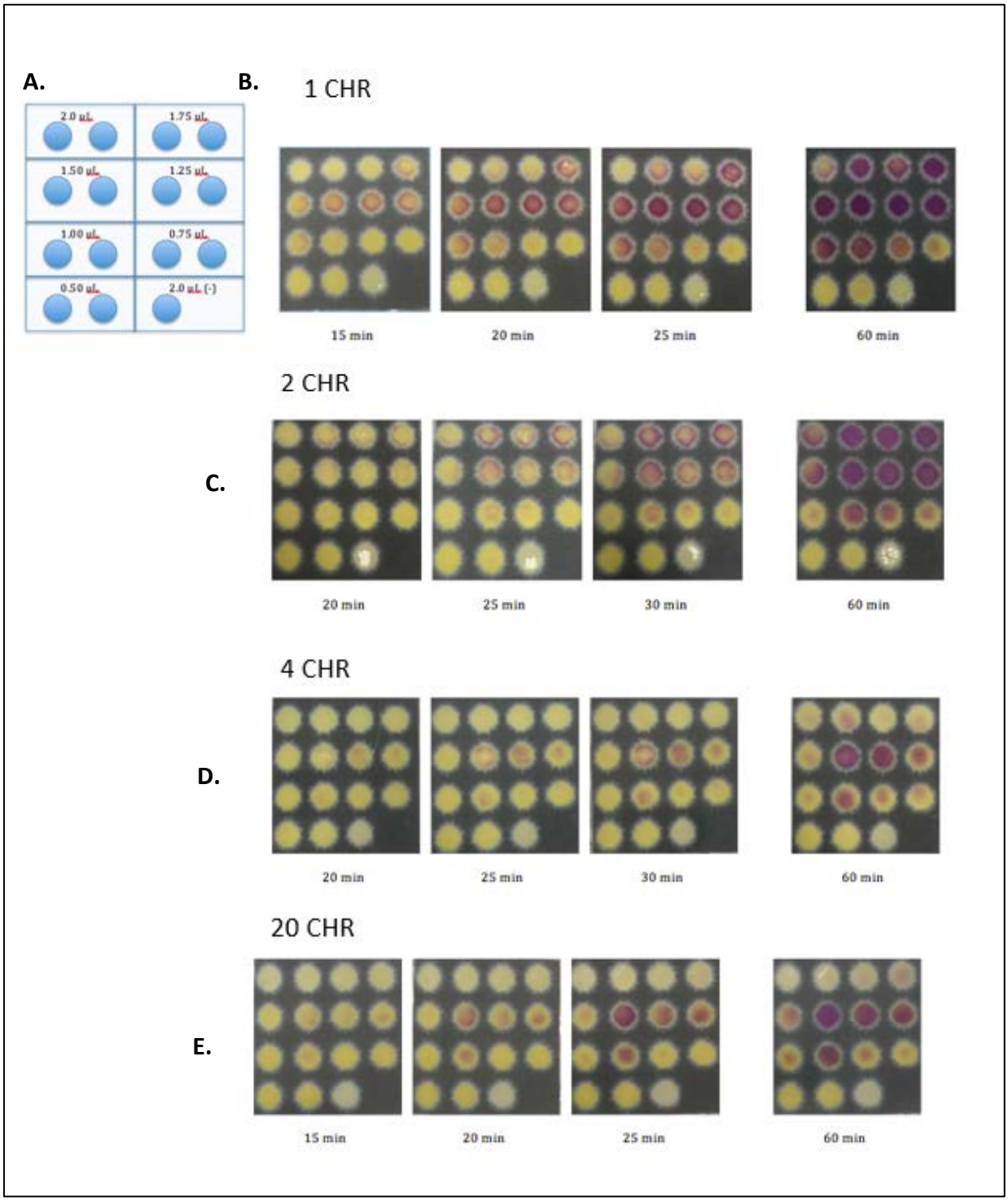


Figure 5. Color change produced on 1 CHR and 3MM papers. The left and right panels show 1 and 4 µL of reaction solutions spotted on the 1 CHR and 3MM papers, respectively. The chosen volumes enabled the reactions to occur when the solutions were totally absorbed within paper.

3.1.5 Rehydration Test Results for Paper

Reaction progression was monitored for 60 min on all paper materials, as displayed in Figure 6A–H. The distributions of various rehydration volumes are shown in the blue diagram in Figure 6A. Generally, a rehydration volume of 1.25 µL seemed to produce the best results in terms of color intensity. Because 2 µL of reaction solution was originally spotted on the paper, the performance of 1.25 µL suggests that a concentration effect enhanced this performance. A rehydration volume as small as 0.75 µL was able to produce a small, but significant color change across all of the paper tickets. It is important to note that wells with equal rehydration volumes often displayed different results. This could be attributed to edge effects because the wells on the upper left edge of the ticket exhibited the most inconsistency. Filter papers 42 (Figure 6F) and 542 (Figure 6G) did not allow the liquid to absorb at the tested rehydration volumes, which indicated that they would not likely be good choices or that there was an in-paper reaction progression. Overall, the 1 CHR paper (Figure 6B) performed the best in terms of color intensity and consistency.



(continued on next page)

Figure 6. Rehydration test. (A) Distribution of various rehydration volumes on paper. Various diH_2O rehydration volumes were tested on (B) 1 CHR, (C) 2 CHR, (D) 4 CHR, (E) 20 CHR, (F) 42, (G) 542, and (H) 3MM papers.

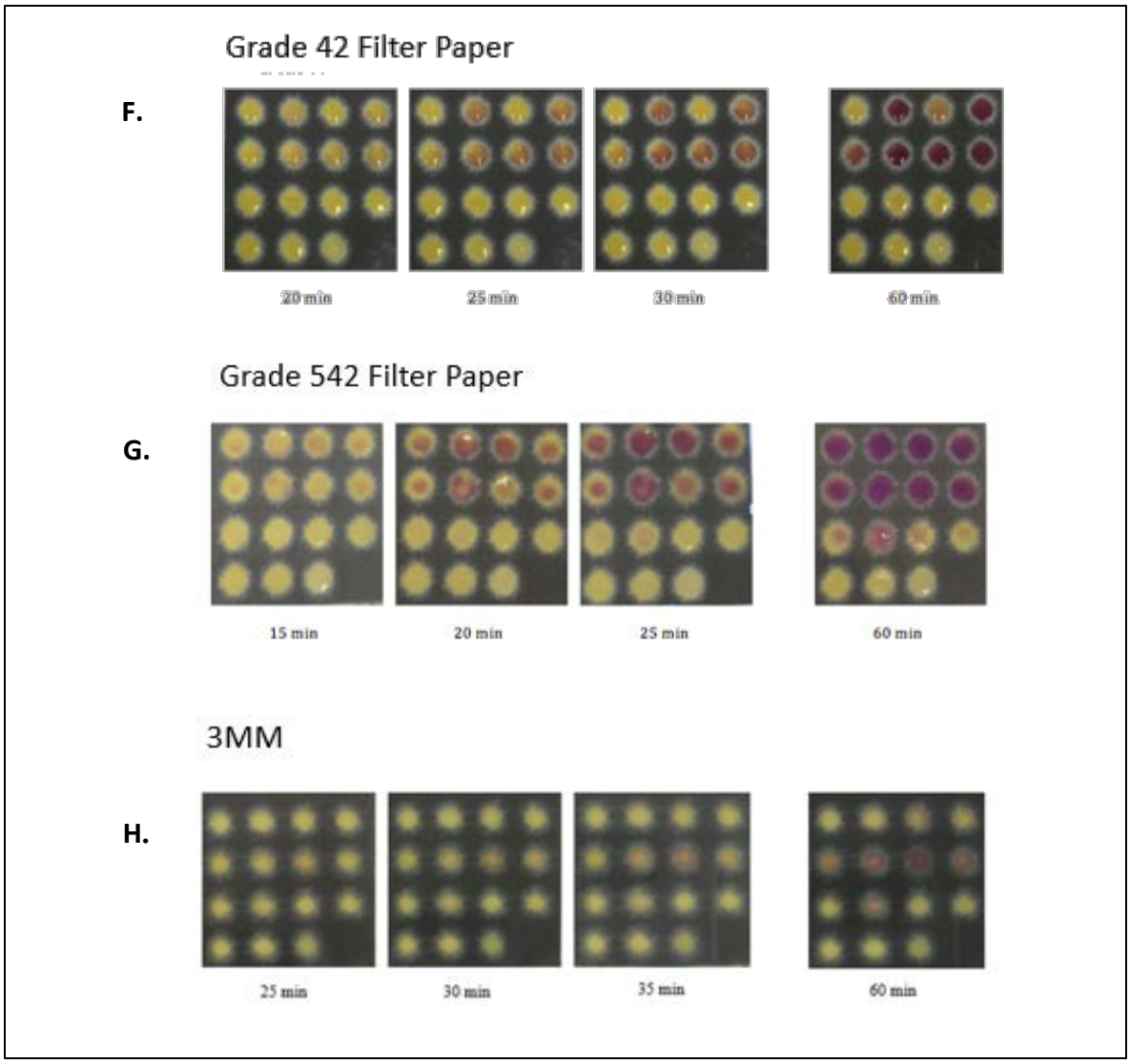


Figure 6. Rehydration test (continued). Various diH_2O rehydration volumes were tested on (B) 1 CHR, (C) 2 CHR, (D) 4 CHR, (E) 20 CHR, (F) 42, (G) 542, and (H) 3MM papers. Reaction progression was monitored for up to 60 min. The 1 CHR paper outperformed the others in terms of color intensity and consistency across wells with equal rehydration volumes.

3.2 Nonpaper and Synthetic Filter Membrane Materials Testing Results

3.2.1 Blocking Test Results for Nonpaper and Synthetic Paper

The unblocked versions of GF/F (GF), GF/B (GB), QM, and TE35 displayed a color change in their membrane centers. However, TE35 failed to absorb the reaction material within its membrane. When materials were blocked with 5% Tween 20, PES, QM, and TE35 exhibited color changes in diffuse patterns across the 6 mm discs. Subsequent testing was therefore performed on unblocked filter membranes because the color displays for these

membranes were more significant. Figure 7 shows the results of the blocking test. The top row of discs in Figure 7 displays unblocked material, and the lower row shows blocked material.

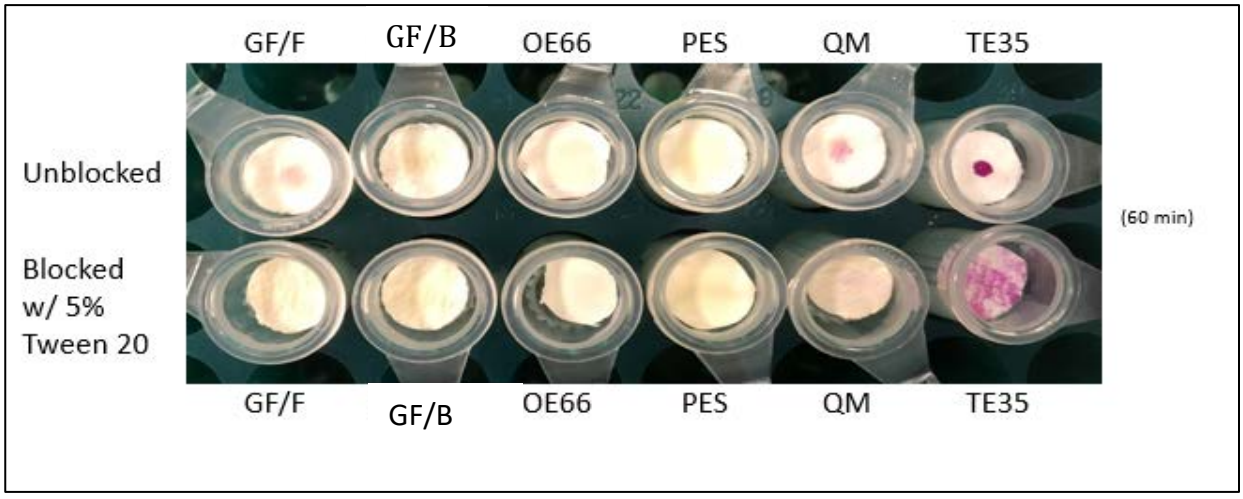


Figure 7. Blocking test results. Blocked (5% Tween 20) and unblocked GF/F (GF), GF/B (GB), OE66, PES, QM, and TE35 materials. Unblocked materials produced a better display of the *lacZ*/CPRG reaction color change.

3.2.2 Preliminary Filter Membrane and Synthetic Paper Comparison Test Results

Among the 13 materials tested, only SP600, SP700, SP800, and iSen exhibited a color change. All materials showed a limited absorption of the reaction solution (Figure 8).

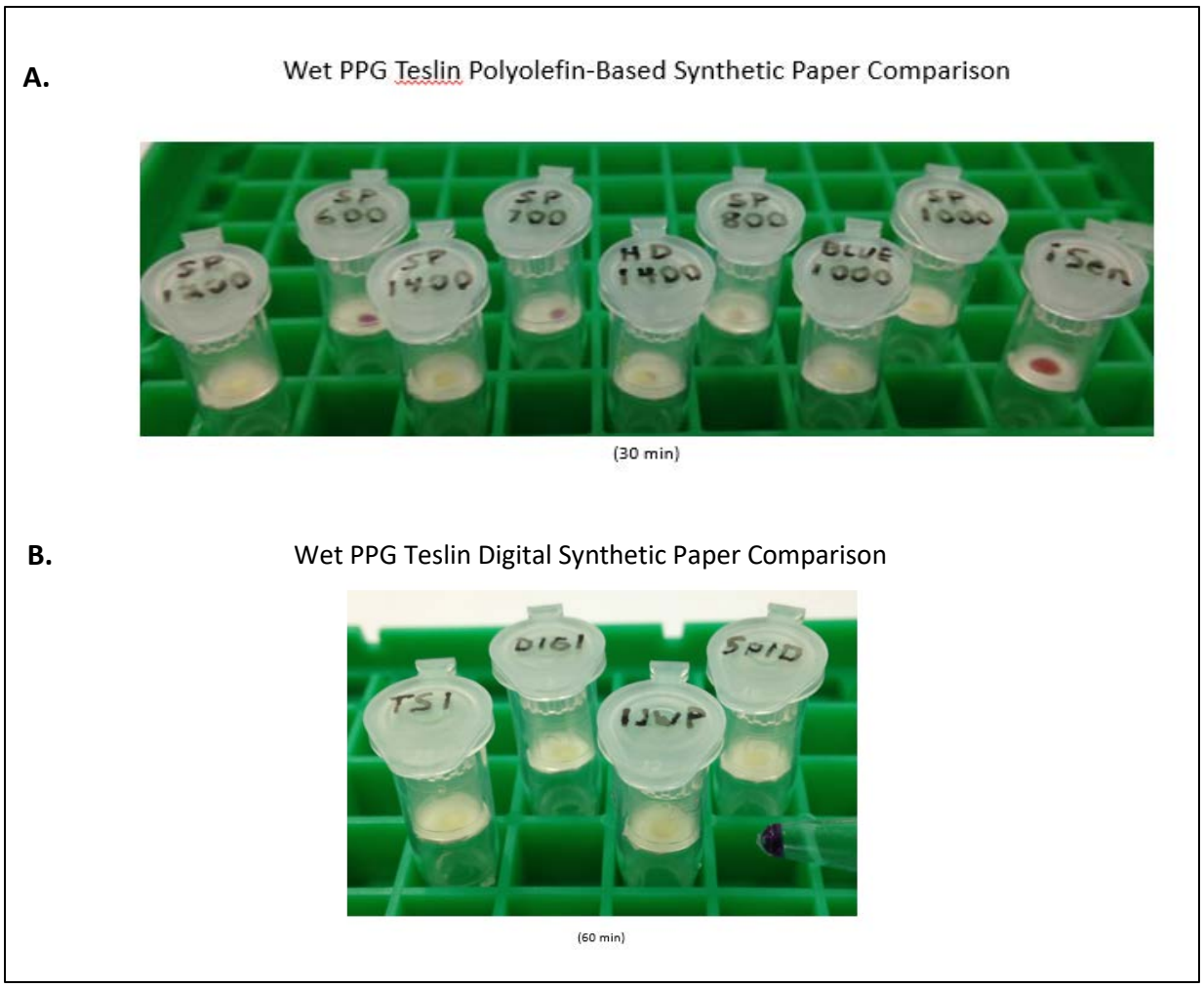


Figure 8. Filter membrane and synthetic paper comparison test. (A) Shows the standard *lacZ*/CPRG reactions on materials with the following sample codes at the 30 min mark, from left to right: SP1200, SP600, SP1400, SP700, HD1400, SP800, BLUE1000, SP1000, and iSen. (B) Shows the rest of the materials used in this test at the 60 min mark: TS1000 (TS1), DIGITAL1000 (DIGI), IJWP1000 (IJWP), and SPID1000 (SPID). SP600, SP700, and iSen showed a change in color, whereas the rest of the materials did not.

3.2.3 Air-Dried Reaction Test Results for Nonpaper and Synthetic Paper

Among the eight materials tested, only TE and iSen displayed color changes at the 30 min mark. The rehydrated reaction solution was completely absorbed in the GB, GF, and QM materials. No absorption was observed in TE, SP600, SP700, SP800, and iSen, as shown in Figure 9A,B.

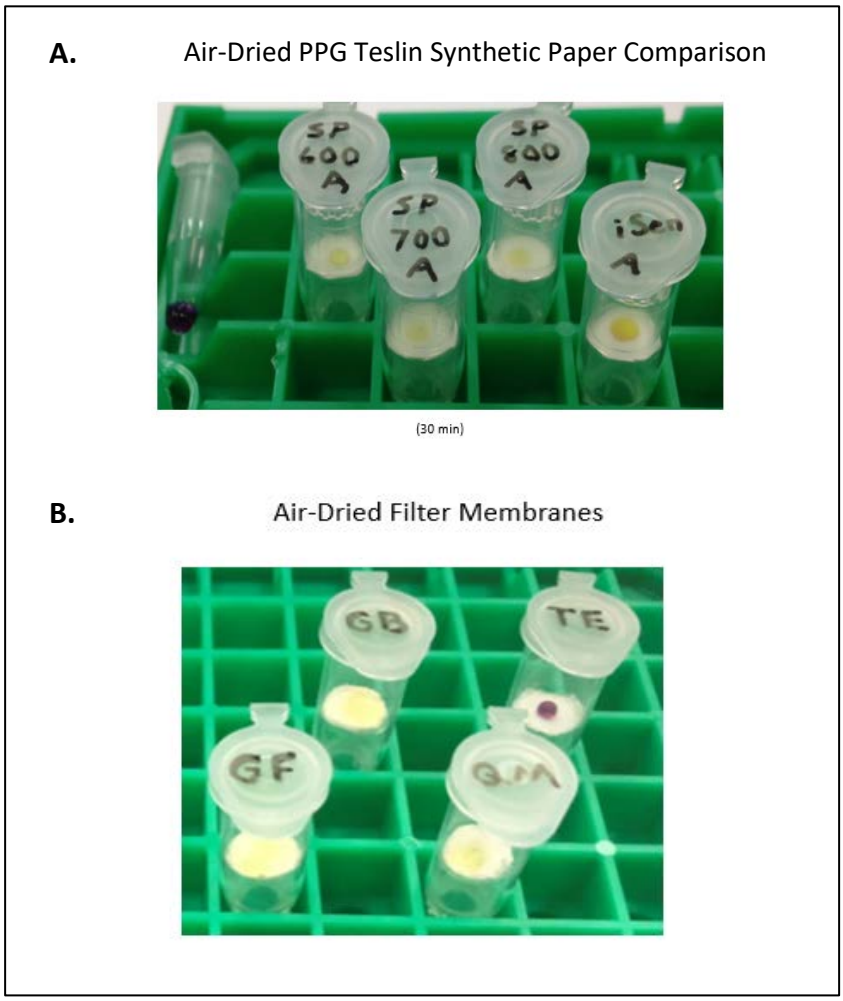


Figure 9. Air-dried reaction test. (A) Shows standard *lacZ*/CPRG reactions that have been air-dried and then rehydrated on materials with the following sample codes at the 30 min mark: SP600, SP700, SP800, iSen. (B) Shows the rest of the materials, GF, GB, TE, and QM, at the 60 min mark. TE and iSen showed a change in color, while the rest of the materials did not.

3.2.4 Air-Dried Reaction Test Results for Nonpaper and Synthetic Paper: Increased Reaction and Rehydration Volume

Among the four materials tested, a color change only occurred in 3MM, as shown in Figure 10. Although it took 1 h for the color change to develop on the edges of the material, a complete color change was observed after 4 h.

Figure 10. Air-dried reaction test with increased reaction and rehydration volume. Color changes were monitored in QM, GB, GF, and 3MM materials in the air-dried test after regular reaction and rehydration volumes were increased from 2.0 µL each to 5.0 and 10 µL, respectively.

3.2.5 Lyophilized Test Results for Nonpaper and Synthetic Paper

This test essentially had the same outcome as the original air-dried test on the filter membranes. Among the eight materials tested, only TE and iSen displayed color changes at the 30 min mark. The rehydrated reaction was completely absorbed in the GB, GF, and QM materials. No absorption was observed in TE, SP600, SP700, SP800, and iSen, as displayed in Figures 11A,B.

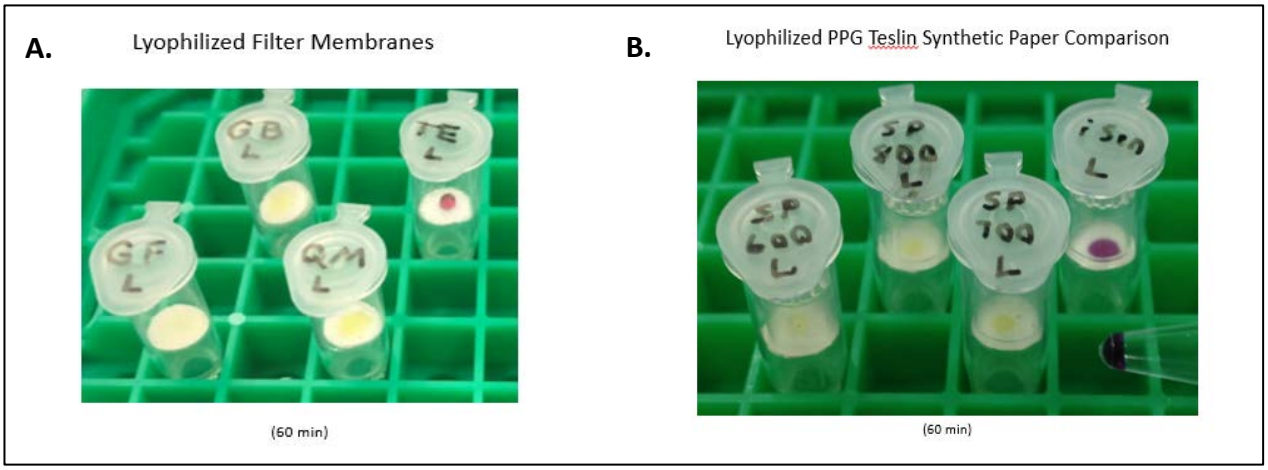


Figure 11. Lyophilized test. (A) Shows *lacZ*/CPRG reactions that have been lyophilized and then rehydrated on materials with the following sample codes at the 60 min mark: GF, GB, QM, and TE. (B) Shows the rest of the materials, SP600, SP800, SP700, and iSen, at the 60 min mark. TE and iSen showed a change in color, whereas the rest of the materials did not.

3.2.6 384-Well Plate Tests Results for Nonpaper and Synthetic Paper

3.2.6.1 Nondried Test Results for Nonpaper and Synthetic Paper

Figure 12 displays the scan images of the 384-well plates at 30 and 60 min into the reaction. The filter membranes and synthetic materials with the following sample codes were included in the plate: GF, GM, PES, QM, TE35, SP60, DIGI, iSen, GA, 934, and Nyln. The paper materials with the following sample codes were included in the plate: PsvU (unblocked), PsvT (blocked 0.05% Tween 20), PsvB (blocked 5% BSA), 42, and 3MM.

The scan was taken on the underside of the disks to show the extent of reaction absorption. PsvB and 3MM showed a quick color change, which started at 20 min. At 25 min, GA had the most significant color change, followed by PsvB and then 3MM. By 60 min, all materials exhibited a notable color change, with the exceptions of Nyln and the negative control, along with a very faint color change in SP60. In terms of absorption of the reaction solution into the various materials, TE35, SP60, DIGI, and Nyln showed no absorption, whereas PES and iSen showed limited absorption. TE35 did, however, show a color change on top of the membrane.

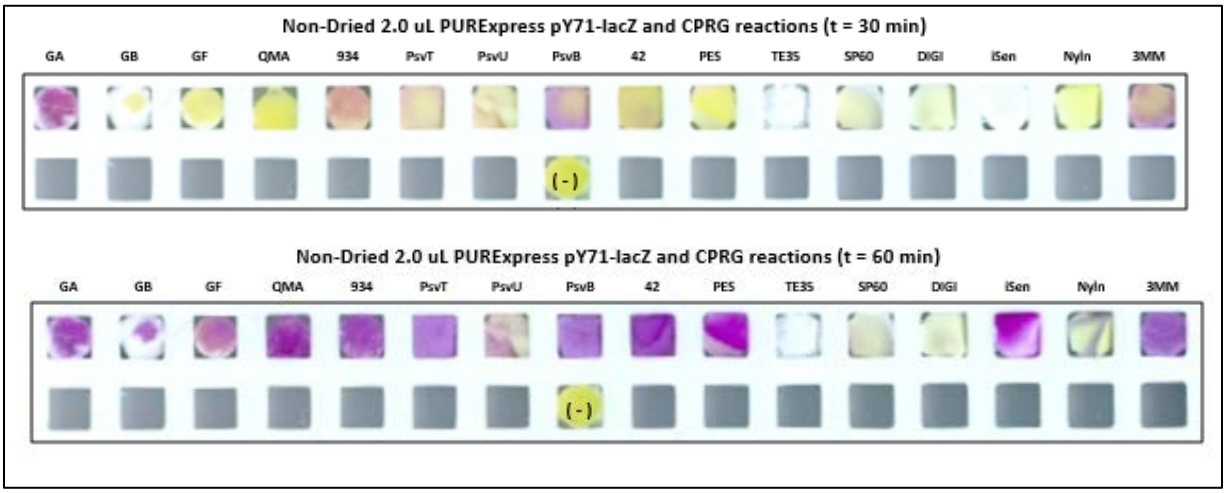


Figure 12. Results for the 384-well plate nondried test with nonpaper and synthetic paper. Wet reaction test in plate format on GA, GB, GF, QMA, 934, PsvT, PsvU, 42, PES, TE35, SP60, DIGI, iSen, Nyln, and 3MM materials at 30 and 60 min into the reaction. By the 60 min mark, a notable color change was seen in all experimental materials with the exception of Nyln.

3.2.6.2 Air-Dried Test Results for Nonpaper and Synthetic Paper

Figure 13 shows the scan images of the 384-well plate containing air-dried tests with nonpaper and synthetic paper at 10, 20, 30, and 60 min into the reaction. The color changes occurred faster under these conditions than those of the nondried test. GA, PsvB, and 934 displayed a color change within 5 min of the reaction start. QMA, 42, and 3MM began turning

color at the 10 min mark. All materials but PES and the negative control exhibited a significant color change by 20 min. The iSen material exhibited a sudden color change after a brief lag period.

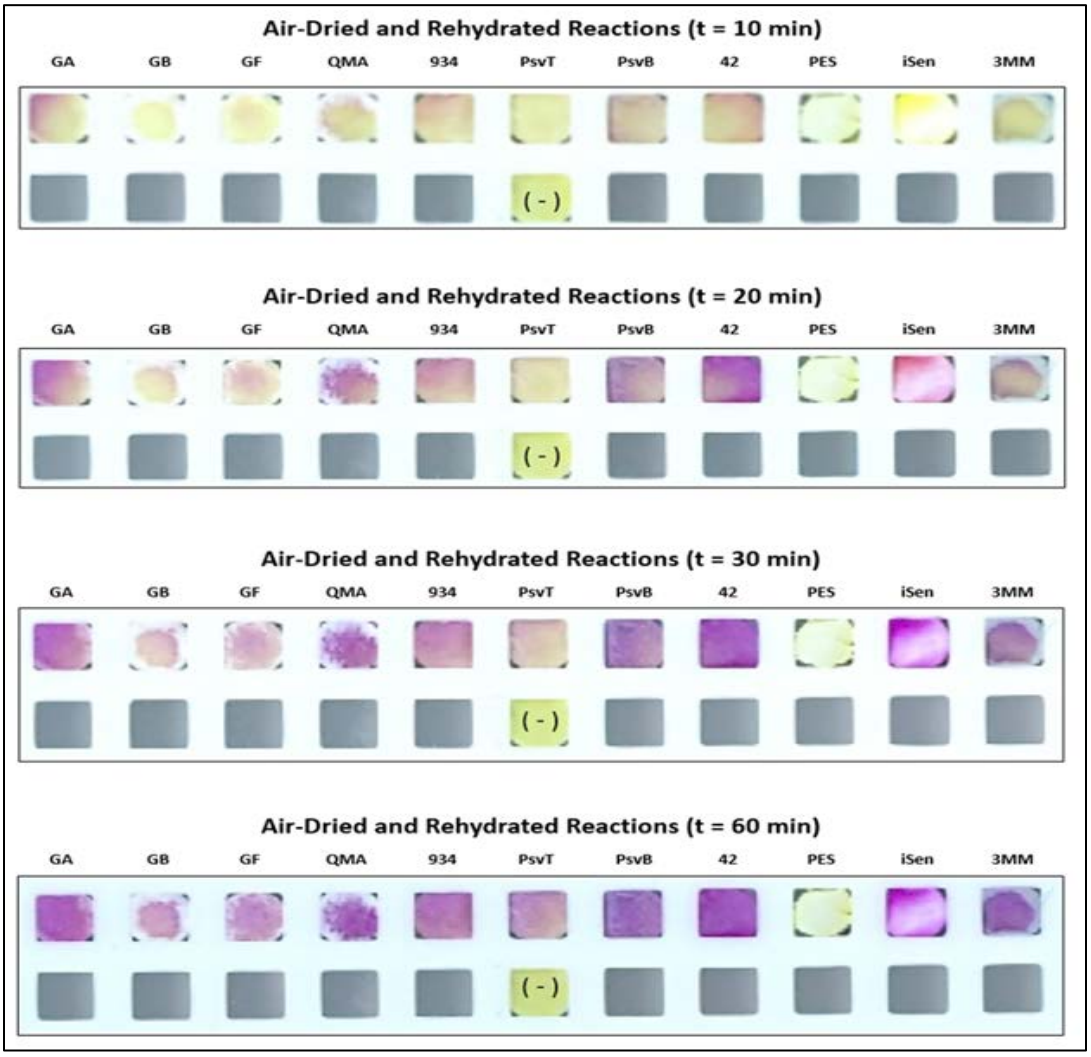


Figure 13. Results for the 384-well plate air-dried test with nonpaper and synthetic paper. Plate air-dried reaction tests on GA, GB, GF, QMA, 934, PsvT, PsvB, 42, PES, iSen, and 3MM materials at 10, 20, 30 and 60 min into the reaction. By the 60 min mark, notable color changes were seen in all experimental materials with the exception of PES.

3.2.6.3 Lyophilized Reaction Test Results for Nonpaper and Synthetic Paper

Figure 14 shows the 384-well plate containing the lyophilized nonpaper and synthetic paper at 25, 35, and 60 min into the reaction. PsvB showed a slight color change at 15 min. PsvB, GA, and QMA showed significant color changes at 20 min, whereas only slight changes were observed in 3MM and GB. No color change was seen in GF, 934, or PES after

60 min. Again, iSen exhibited a rapid color change after a lag period. Limited absorption was noted in PES and iSen.

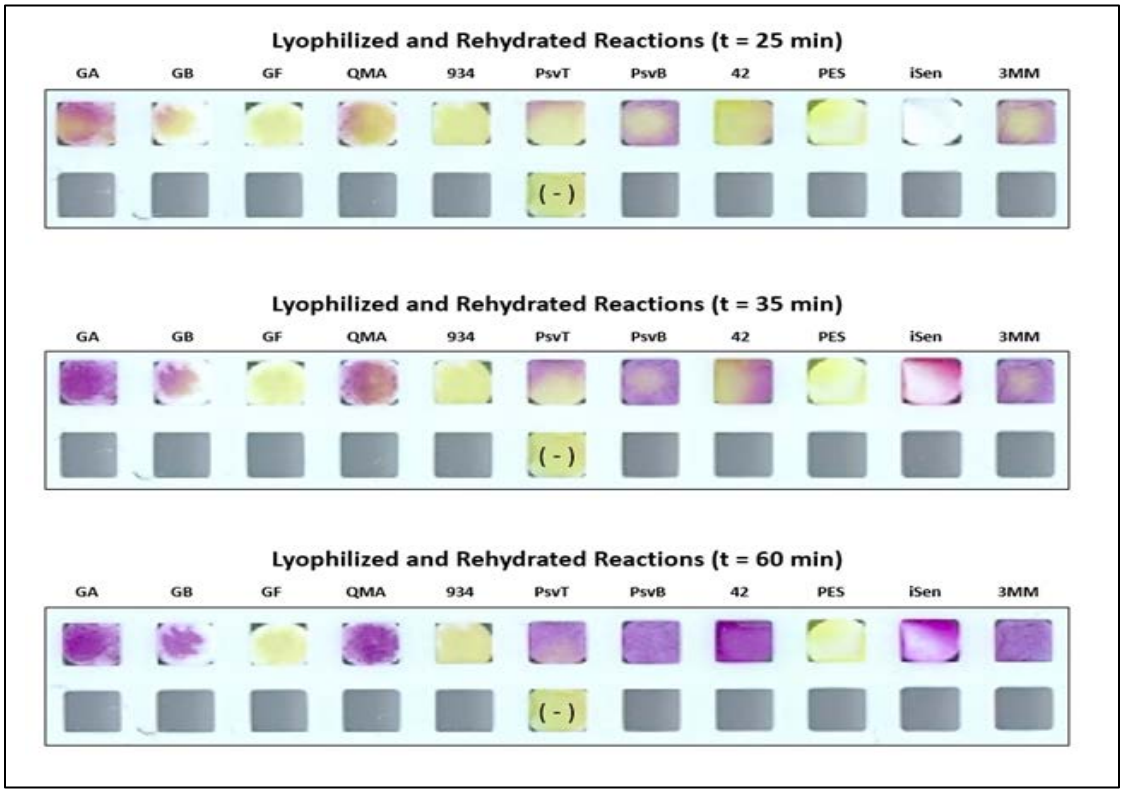


Figure 14. Results for the 384-well plate lyophilized test with nonpaper and synthetic paper. Lyophilized plate test on GA, GB, GF, QMA, 934, PsvT, PsvB, 42, PES, iSen, and 3MM materials at 25, 35, and 60 min into the reaction. By the 60 min mark, notable color changes were seen in all experimental materials with the exceptions of GF, 934, and PES.

4. CONCLUSIONS

The preliminary results displayed in this document suggest that our quest to find suitable paper and filter membrane materials for the bases of cell-free detection systems shows great promise. First, the viability of the pY71_*lacZ* constructs was confirmed, and reactions were run to completion in a variety of wet and dry conditions. Through multiple volume, rehydration, wet reaction, dry reaction, and lyophilized reaction tests, several materials demonstrated that they are suitable for running *lacZ*/CPRG reactions and that they allow for the development of a notable color change. We noted that the blocking of some materials, such as the paper materials, with BSA enhanced the surface characteristics for the cell-free reactions. In other cases, blocking with materials such as Tween 20 produced negative results at 5% and positive results at 0.05%, as was shown in the synthetic filter membrane blocking test and the nondried plate test. Additional optimization for the promising material types is needed due to the significant variations that we observed regarding color intensity and reaction time. Several

factors of the reaction can be targeted for improvement, including reaction and rehydration volumes, well sizes, and reporter/substrate pairs other than *lacZ*/CPRG. Additionally, some materials may need to incorporate wells to allow for hydration and for successful reaction progression. This is because some materials, such as glass and quartz microfiber, did not work well when relatively large surface areas were involved; although they have worked in previous studies.

Among the many materials tested, those with the following sample codes are likely to advance for subsequent testing based on their ability to allow the reaction to develop fully and in a timely manner: all paper materials, including 1 CHR, 2 CHR, 4 CHR, 20 CHR, 3 MM, 42, 542, and Psv, and the synthetic filter membranes GA, GB, GF, QMA, and iSen. Implementation of new imaging methods will allow time-course data to be collected and help to decide which materials will be best for our purposes. It will then be possible to determine more accurately the materials that exhibit the fastest color changes and those that produce the most intense colors.

Finally, in future studies, we hope to expand our collection of reporters and substrates to allow for the possibility of multiplexing, wherein testing for multiple substances on the same ticket can be performed.

Blank

LITERATURE CITED

1. Khalil, A.S.; Collins, J.J. Synthetic Biology: Applications Come of Age. *Nat. Rev. Genet.* **2010,** *11* (5), 367–379.

2. Pardee, K.; Green, A.A.; Ferrante, T.; Cameron, D.E.; DaleyKeyser, A.; Yin, P.; Collins, J.J. Paper-Based Synthetic Gene Networks. *Cell* **2014,** *159* (4), 940–954.

Blank

ACRONYMS AND ABBREVIATIONS

bp	base pair
BSA	bovine serum albumin
CPRG	chlorophenol red-β-D-galactopyranoside
diH_2O	deionized water
DNA	deoxyribonucleic acid
DoD	U.S. Department of Defense
LacZ	β-galactosidase (protein)
lacZ	β-galactosidase gene designation
NA	not applicable
NEB	New England BioLabs, Inc.
PCR	polymerase chain reaction
PE	polyethylene
RNase	ribonuclease

DISTRIBUTION LIST

The following individuals and organizations were provided with one Adobe portable document format (pdf) electronic version of this report:

U.S. Army Edgewood Chemical Biological Center (ECBC), BioChemistry Branch
RDCB-DRB-C
ATTN: Blum, S.
Funk, V.
Lux, M.
Chue, C.

Defense Threat Reduction Agency, R&D
J9-CBS
ATTN: Cronce, D.

Department of Homeland Security
RDCB-PI-CSAC
ATTN: Mearns, H.

Defense Technical Information Center
ATTN: DTIC OA

G-3 History Office
U.S. Army RDECOM
ATTN: Smart, J.

ECBC Technical Library
RDCB-DRB-BL
ATTN: Foppiano, S.
Stein, J.

Office of the Chief Counsel
AMSRD-CC
ATTN: Upchurch, V.

ECBC Rock Island
RDCB-DES
ATTN: Lee, K.
RDCB-DEM
ATTN: Grodecki, J.


EDGEWOOD CHEMICAL BIOLOGICAL CENTER
A U.S. Army RDECOM Laboratory