An automated and high-throughput polymerization solution for downstream tissue clearing: the X-CLARITY™ Polymerization System

Introduction

The tissue clearing method CLARITY transforms intact tissues into tissue-hydrogel hybrids from which lipids are removed to make structurally stable and optically transparent tissues. A key step of the CLARITY method is creating the tissue-hydrogel hybrid to physically support the tissue ultrastructure and preserve molecular information during the lipid clearing process. Tissues are incubated in a solution of acrylamide and VA-044, a thermal initiator. At higher temperatures (e.g. 37°C), VA-044 decomposes in solution and releases free radicals. Free radicals initiate the polymerization of hydrogel monomers. The process is done in a strictly anaerobic environment, as oxygen is highly reactive with free radicals and consequently strongly inhibits polymerization.

The conventional protocol involves removing oxygen from the sample container using a vacuum pump, desiccation chamber, and a nitrogen gas supply, after which the sample must be heated to activate the initiator. The precision required and user error involved increases the chance of improperly cleared tissues downstream and also limits the number of tissue samples that may be processed simultaneously. Such factors add to the variability of test results due to inconsistent experimental conditions and/or operator judgement. The X-CLARITY™ Polymerization System was developed to standardize the polymerization process by improving experimental consistency and reliability with less time and effort.

X-CLARITY™ Polymerization System

The X-CLARITY™ Polymerization System (Figure 1) is a standalone, automated system developed to standardize and simplify hydrogel-tissue hybridization. It can efficiently process up to 768 samples simultaneously. Conventional hydrogel polymerization requires multiple steps, including degassing the sample in a desiccator chamber with a vacuum pump, replacing the oxygen with an inert gas, and then heating the sample in a shaking incubator or water bath.

The X-CLARITY™ Polymerization System is an all-in-one solution that eliminates the need for extra equipment. Multi-well plates or conical tubes can be placed directly in the system for rapid and efficient high-throughput sample processing. Users can control polymerization by adjusting vacuum strength, temperature, and a timer through a simple touchscreen interface. (Figure 2).

Figure 1. The X-CLARITY™ Polymerization System

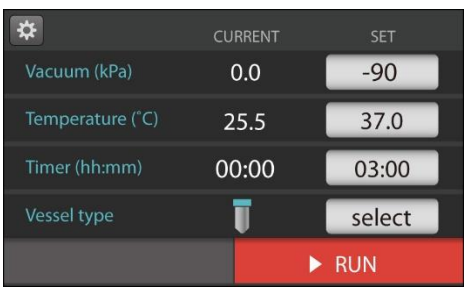

Figure 2. Touchscreen interface.
The simple touchscreen interface of the X-CLARITY™ Polymerization System gives users control over vacuum pressure, temperature, and polymerization time.

Effective temperature for polymerization

The 10-hour half-life decomposition temperature of VA-044 is 44°C, but this temperature is potentially too high to ensure protein stability in tissue-hydrogel hybrids. To test for the ideal polymerization temperature, hydrogel solution samples (4% acrylamide, 0.05% *bis*-acrylamide) were polymerized at 35, 37, and 44°C for 3 hours at -90 kPa with the X-CLARITY™ Polymerization System. 35°C was sufficient for VA-044 activation and subsequent hydrogel polymerization. Although there was little difference in polymerization efficiency for the different temperatures, gels polymerized at 44°C had large bubbles form within the gel (Figure 3). Bubble formation may affect the formation of a uniform tissue-hydrogel network. The temperature control of the X-CLARITY™ Polymerization System helps ensure polymerization consistency between samples.

Time required for polymerization

To determine optimal polymerization times, hydrogel solution samples (4% acrylamide, 0.05% *bis*-acrylamide) were polymerized with the conventional method and in the X-CLARITY™ Polymerization System. For the conventional method, the sample containers were placed in a desiccation chamber. The chamber was degassed with a vacuum pump and then flooded with nitrogen gas. The sample containers were sealed and placed in a 37°C shaking incubator for 1, 2, or 3 hours. For the X-CLARITY™ Polymerization System, sample containers were placed in the system and run at 37°C for 1, 2, or 3 hours at -90 kPa. The X-CLARITY™ Polymerization System nearly completely polymerized samples within one hour, whereas the conventional method took longer (Figure 4).

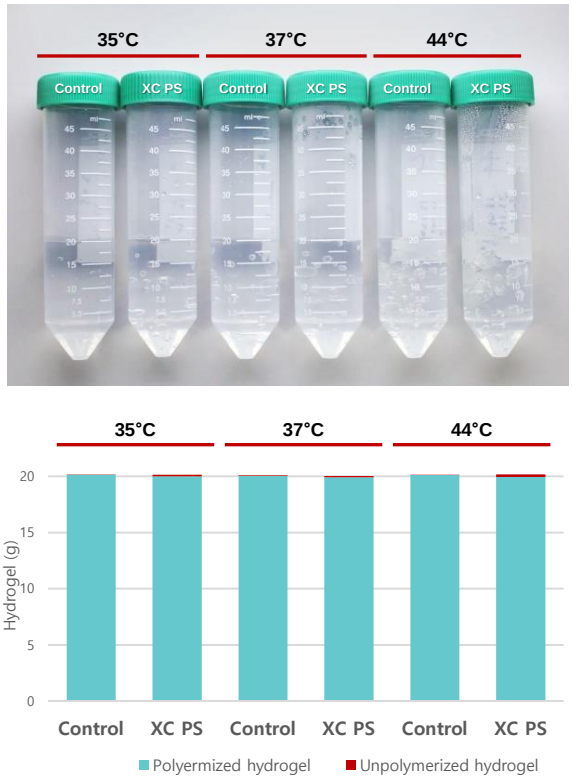

Figure 3. The effects of temperature on polymerization.
Both the X-CLARITY™ Polymerization System (XC PS) and the conventional method (Control) polymerized the hydrogel solution efficiently at 35, 37, and 44℃.

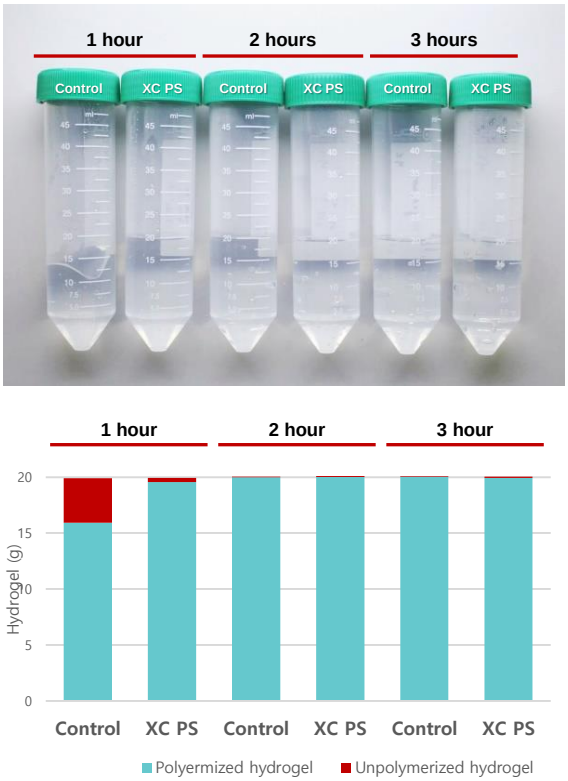

Figure 4. The effects of time on polymerization.
The X-CLARITY™ Polymerization System (XC PS) polymerized the hydrogel solution faster than the conventional method (Control), taking just 1 hour to completely polymerize the hydrogel solution. There was little difference between the two methods after 2 hours.

Polymerization consistency

Additional experiments were conducted to test whether there was a difference in polymerization depending on sample location within the X-CLARITY™ Polymerization System chamber. A 96-well plate containing hydrogel solution (4% acrylamide, 0.05% *bis*-acrylamide) was placed in the X-CLARITY™ Polymerization System and the system was run at 37°C for 3 hours at -90 kPa. Location had no effect on polymerization efficiency and there was consistent polymerization from well to well (Figure 5A). The test was repeated with a 384-well plate, producing the same results (Figure 6).

The results were further confirmed by immunolabeling tissues that were polymerized with the X-CLARITY™ Polymerization System. Adult mouse brain cortex samples were incubated in a *bis*-acrylamide-free hydrogel solution (4% acrylamide) for 24 hours at 4°C. The samples were placed in the X-CLARITY™ Polymerization System and the system was run at 37°C for 3 hours at -90 kPa.

The samples were electrophoretically cleared with the X-CLARITY™ Tissue Clearing System and then washed with 1X PBS for 15 hours at RT. The samples were incubated with anti-collagen IV antibody (1:400, Abcam) in 1X PBS containing 6% BSA and 0.2% Triton X-100 at 37°C for 24 hours. After washing with 1X PBST for 24 hours at 37°C, the samples were incubated with Cy3-conjugated secondary antibody (1:250, Jackson ImmunoResearch Laboratories) for 24 hours at 37°C. After washing with 1X PBST for 24 hours at 37°C, the samples were incubated in X-CLARITY™ Mounting Solution (C13101, Logos Biosystems) at RT for 3 hours.

Images were acquired with a Zeiss LSM 710 (Carl Zeiss) using a 10X Plan Neofluar objective lens and analyzed with ZEN software (Carl Zeiss). Images confirmed that the X-CLARITY™ Polymerization System efficiently processes multiple samples regardless of sample placement within the chamber (Figure 5B).

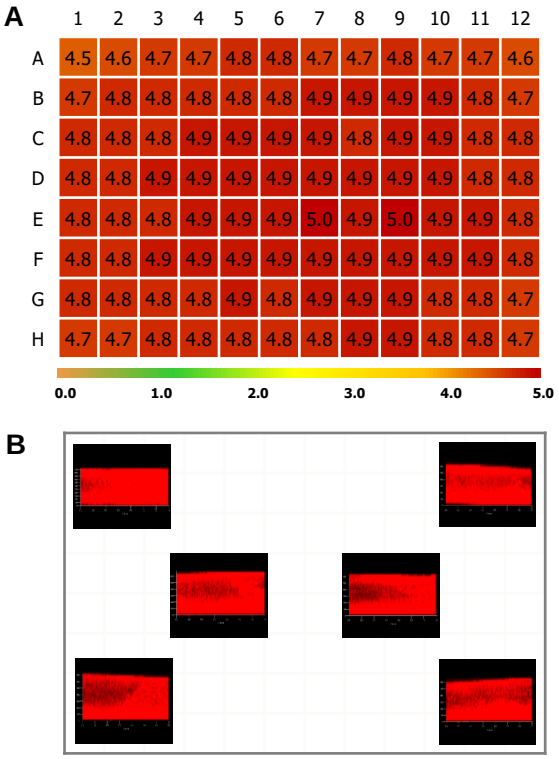

Figure 5. Polymerization test of different locations on a 96-well plate.
(A) There was little variation well to well. Relative scores were determined by comparing the weight of the gels in each well to the heaviest gel. The relative score of the heaviest gel was set to 5.0. (B) Immunolabeling showed little difference between the samples polymerized in different wells. Z-stack images were obtained from a mouse brain cortex samples labeled with anti-collagen IV.

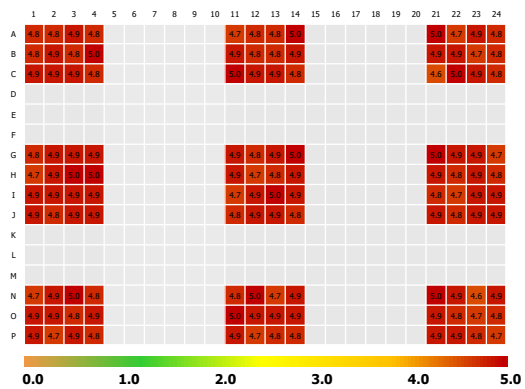

Figure 6. Polymerization test of different locations on a 384-well plate.
There was little variation well to well.

	Conventional method	X-CLARITY™ Polymerization System
Equipment	Vacuum pump Desiccation chamber Nitrogen tank Shaking incubator or water bath	X-CLARITY™ Polymerization System
Control mechanism	Manual Multistep protocol	Fully automated Simple touchscreen interface
Compatible vessels	Conical tubes	Conical tubes Multiwell plates
Throughput	Low	High *up to 768 samples
Time control	Manual	Automated
Vacuum control	Manual Analog	Automated Digital
Polymerization efficiency	Low	High

Table 1. Comparison between the conventional polymerization method and the X-CLARITY™ Polymerization System.

Summary

These comparisons demonstrate that the X-CLARITY™ Polymerization System is a reliable and simple alternative to the laborious manual polymerization protocol (Table 1). The benefits of automating the polymerization process include effortless oxygen removal, consistent temperature control, uniform polymerization, and standardization of the polymerization step for downstream tissue clearing. The system can also efficiently process multiple samples simultaneously. The X-CLARITY™ Polymerization System and X-CLARITY™ Tissue Clearing System are important technological advances in standardizing tissue clearing and provides the researcher a rapid, streamlined solution for processing tissues for volumetric imaging.

Find out more at www.logosbio.com

Ordering Information

X-CLARITY™ Polymerization System			
Starter Kit	C20001	X-CLARITY™ Polymerization System	1 unit
Components	C20002	X-CLARITY™ Heat Block for 6 x 50 mL tubes	1 unit
	C20003	X-CLARITY™ Heat Block for flat-bottom plates	1 unit
Reagents	C1310X	X-CLARITY™ Hydrogel Solution Kit	1 box
	C13103	X-CLARITY™ Hydrogel Solution	1 L
	C13104	X-CLARITY™ Polymerization Initiator	2.5 g
X-CLARITY™ Tissue Clearing System			
Starter Kit	C10001	X-CLARITY™ Tissue Clearing System	1 unit
Reagents	C13001	Electrophoretic Tissue Clearing Solution	12 x 1 L
	C13101	X-CLARITY™ Mounting Solution	1 x 25 mL
	C13102	X-CLARITY™ Mounting Solution Value Pack	10 x 25 mL

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