CATCH Guide for SageHLS System

March, 2018

Overview of HLS-CATCH Process

1) Prior to day of extraction, schedule availability of cells. Check that cell preparation reagents are ready.

2) Prepare cells using Sage HLS cell prep kit instructions (kits/protocols for mammalian WBCs, tissue culture cells, and bacterial spheroplasts are available). Recommended cell load will contain ~2.5 ug genomic DNA per lane (for example, ~375,000 human diploid cells per lane) in a maximum loading volume of 70 ul. Higher cell loads will result in lower recovery of genomic CATCH targets.

3) Prepare HLS cassettes for run. (Dislodge bubbles, open cassettes, empty and refill elution modules, fill electrode reservoirs with electrophoresis buffer to top edge of cassette. Perform current test.)

4) Assemble wild-type SpCas9 enzyme with gRNAs. (Assembly can be done in advance.)

5) Set up desired workflow in software.

6) Load samples and carry out run, including user interventions for Treatment Stage using customized Cas9 cleavases.

7) After run, remove tape seals and remove samples from elution modules, preferably using wide-bore pipette tips.

HLS Software for CATCH

General description of HLS software

Users will use (and create) “Workflows” comprising up to three “Stages”.

The “Stages” fall into the following categories:

Stage 1 - Extraction

Cells are lysed by a brief period of DC electrophoresis (1 or 3 hrs). Released HMW genomic DNA enters the agarose wall of the sample well, and becomes immobilized there because of its large size (>10mb).

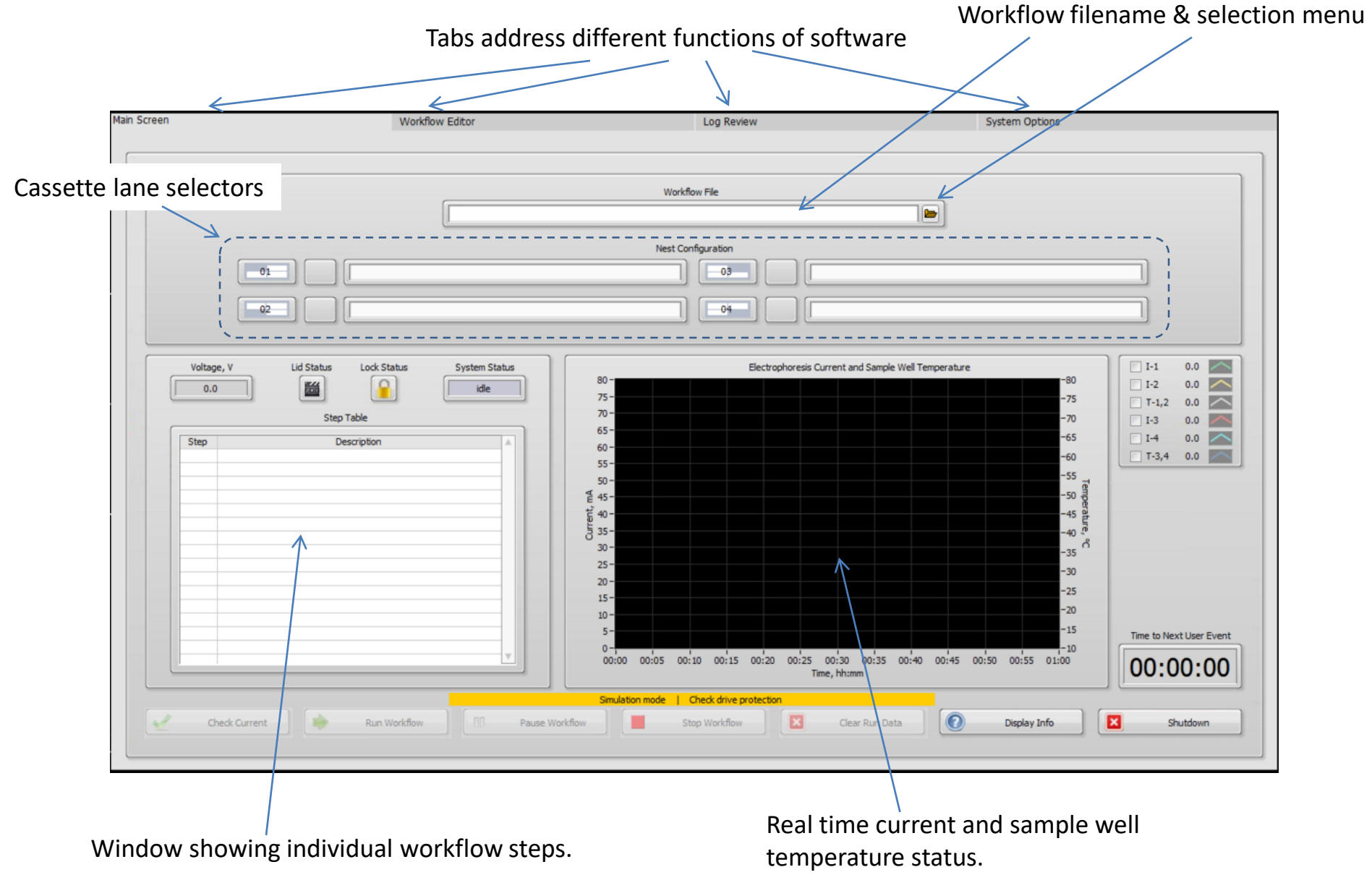
Stage 2 – Treatment

The user adds customized Cas9 cleavases to the sample well to release genomic region(s) of interest from immobilized genomic DNA in wall of sample well.

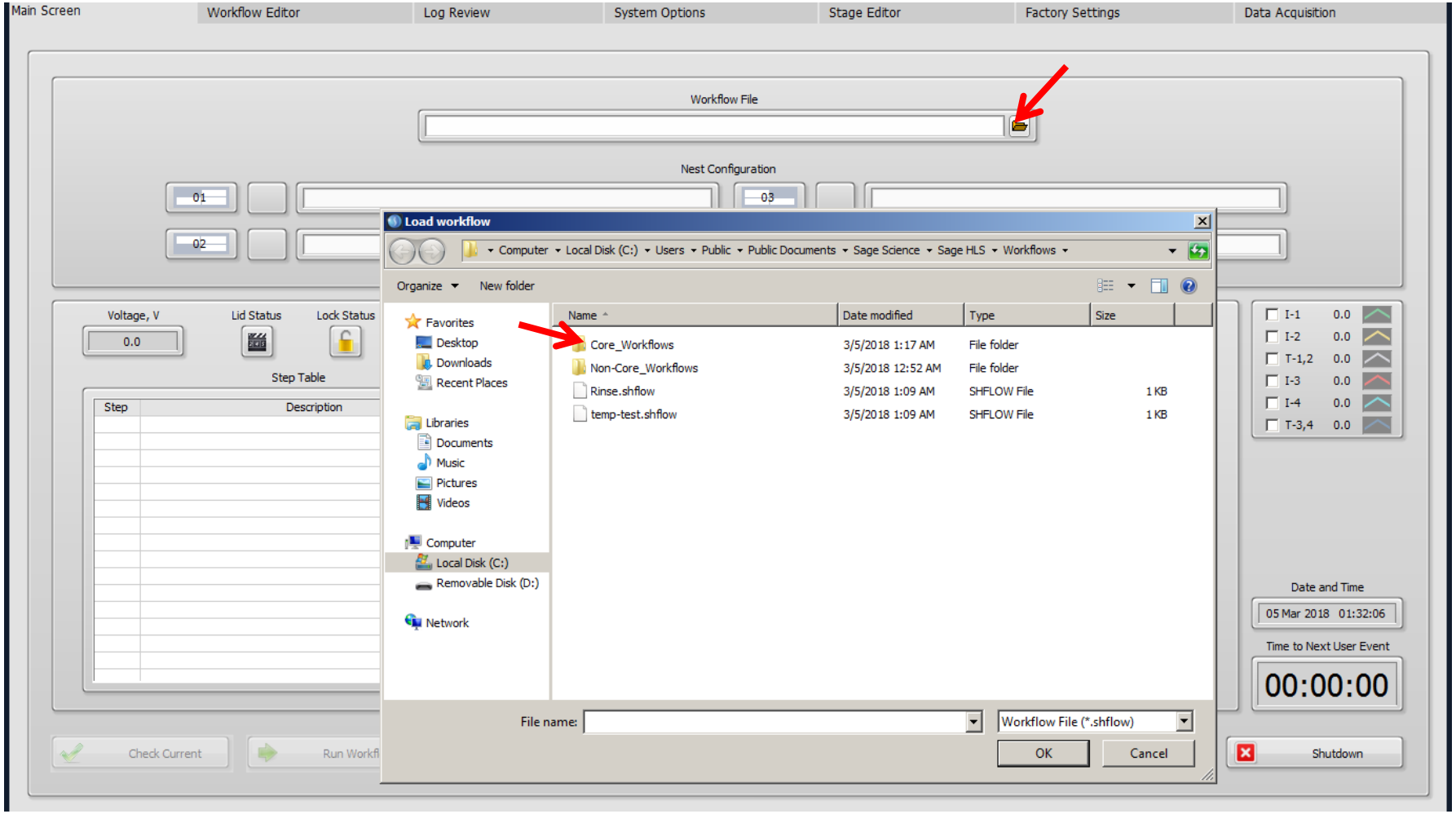
Stage 3 – Collection

Electrophoretic size selection is carried out to remove enzymatic reagents, separate the targeted genomic fragments from non-specific digestion products, and electroelute the products into the elution modules.

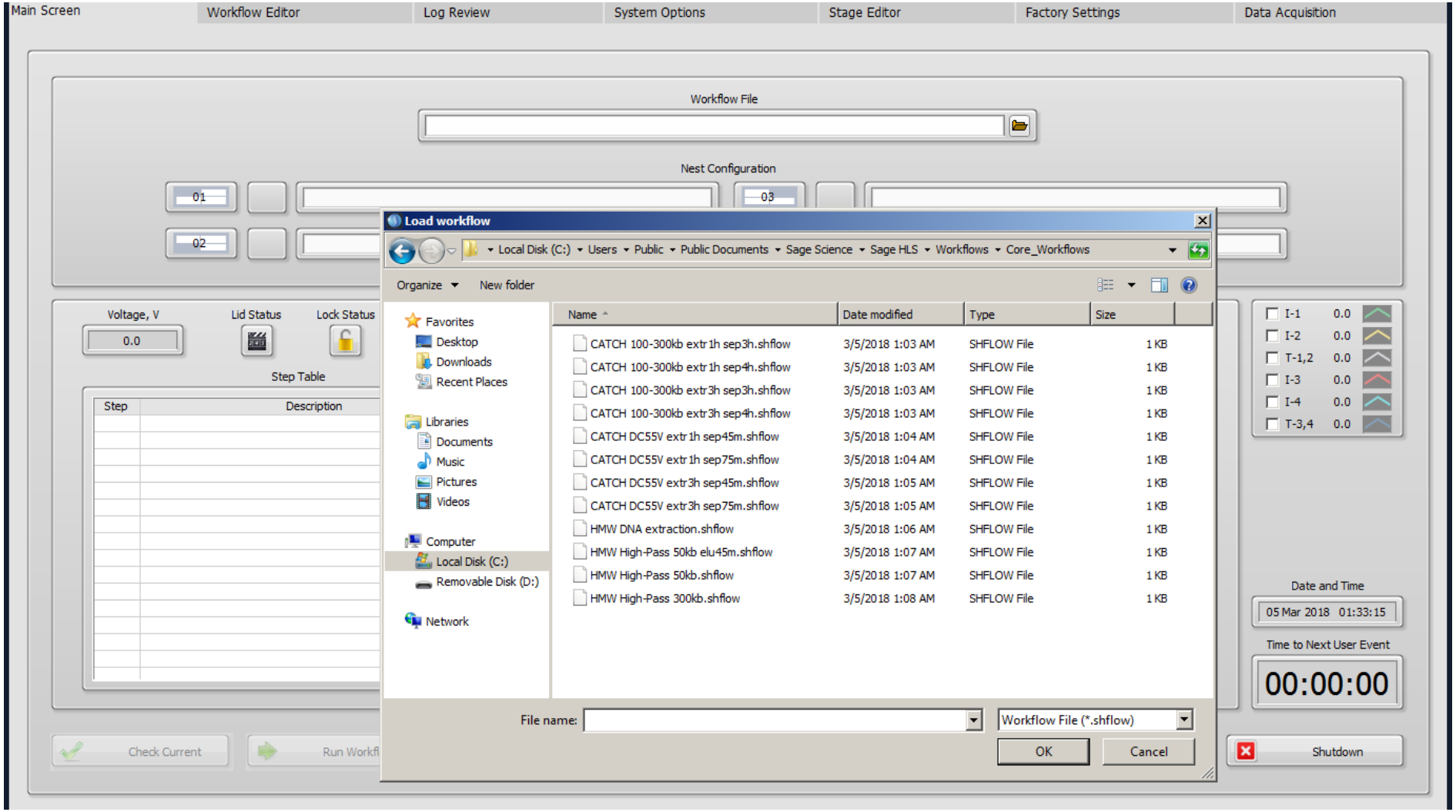
Main screen on instrument startup



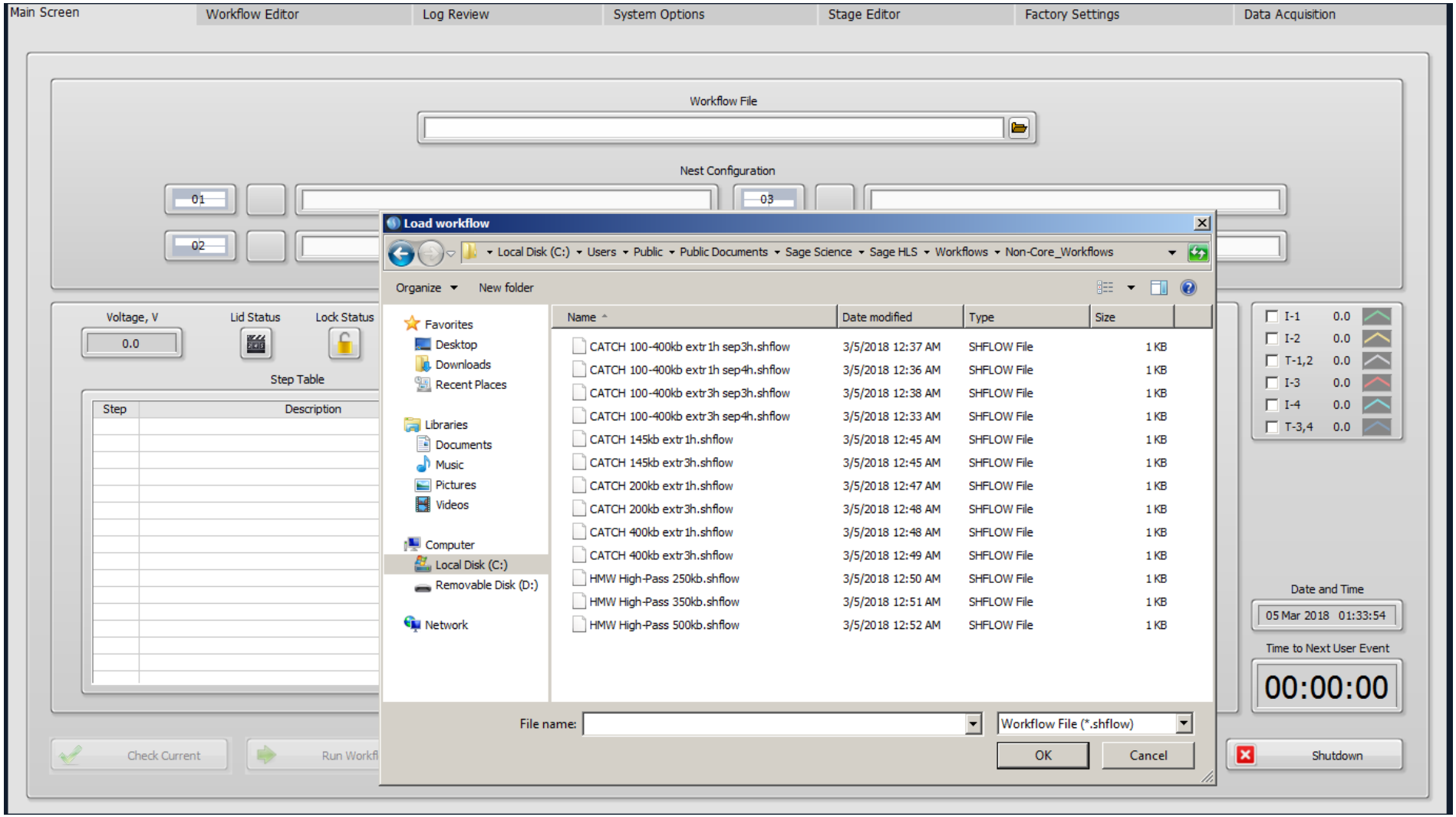
Workflows can be loaded from the “Main Screen” by clicking on the Workflow file directory icon, which will open a Workflow directory window. The Workflows are grouped into two categories, “Core” and “Non-core”, based on how much experience Sage has had with the workflow. Double-click on the folder to see the workflow files. Workflow files have a “.shflow” filetype.



Workflows designed for HLS-CATCH experiments include **“CATCH”** as the first term. Workflows designed for HMW DNA extraction include **“HMW”** as the first term. Some of the workflows share size-selection electrophoresis conditions, but differ in the duration of the extraction and/or collection stages. For instance the workflow “CATCH 100-300kb **extr1h sep3h**” uses a 1 hour extraction stage, and a 3 hour size-separation collection stage. The “CATCH 100-300kb **extr3h sep4h** uses the same collection waveform (“100-300kb”), but uses a 3hr extraction stage, and a 4 hour collection stage.

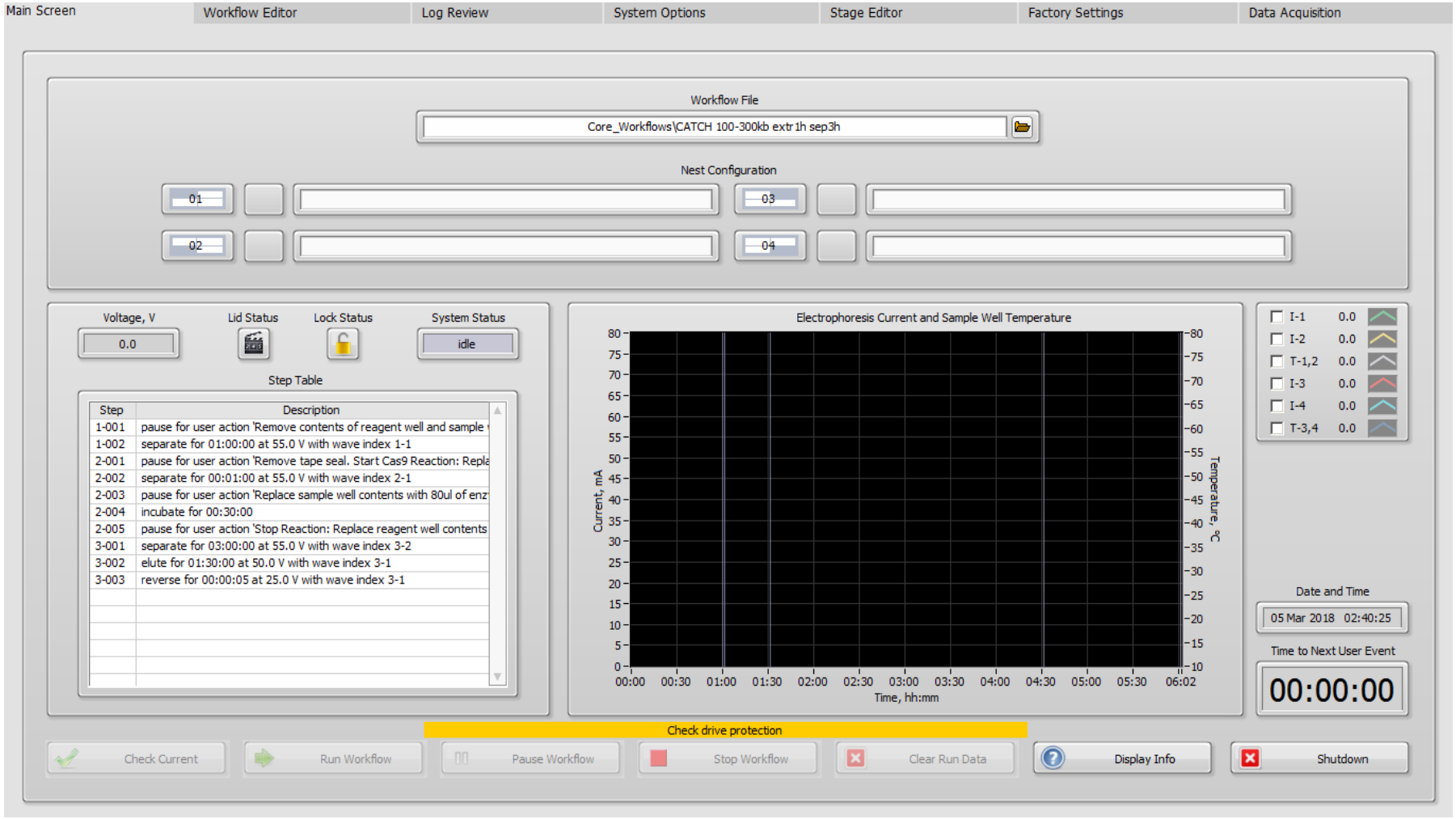


<u>Non-core workflows</u> have been designed for special purposes. For instance, the **CATCH 100-400k** workflows are designed to have a broader collection range than the core 100-300kb workflows, whereas the "**CATCH <xxx>kb**" workflows are designed to have expanded resolution in a <u>narrow window</u> centered on <xxx>kb. In general, Sage has tested the non-core workflows with model samples, but doesn't yet have extensive test data from biological samples.



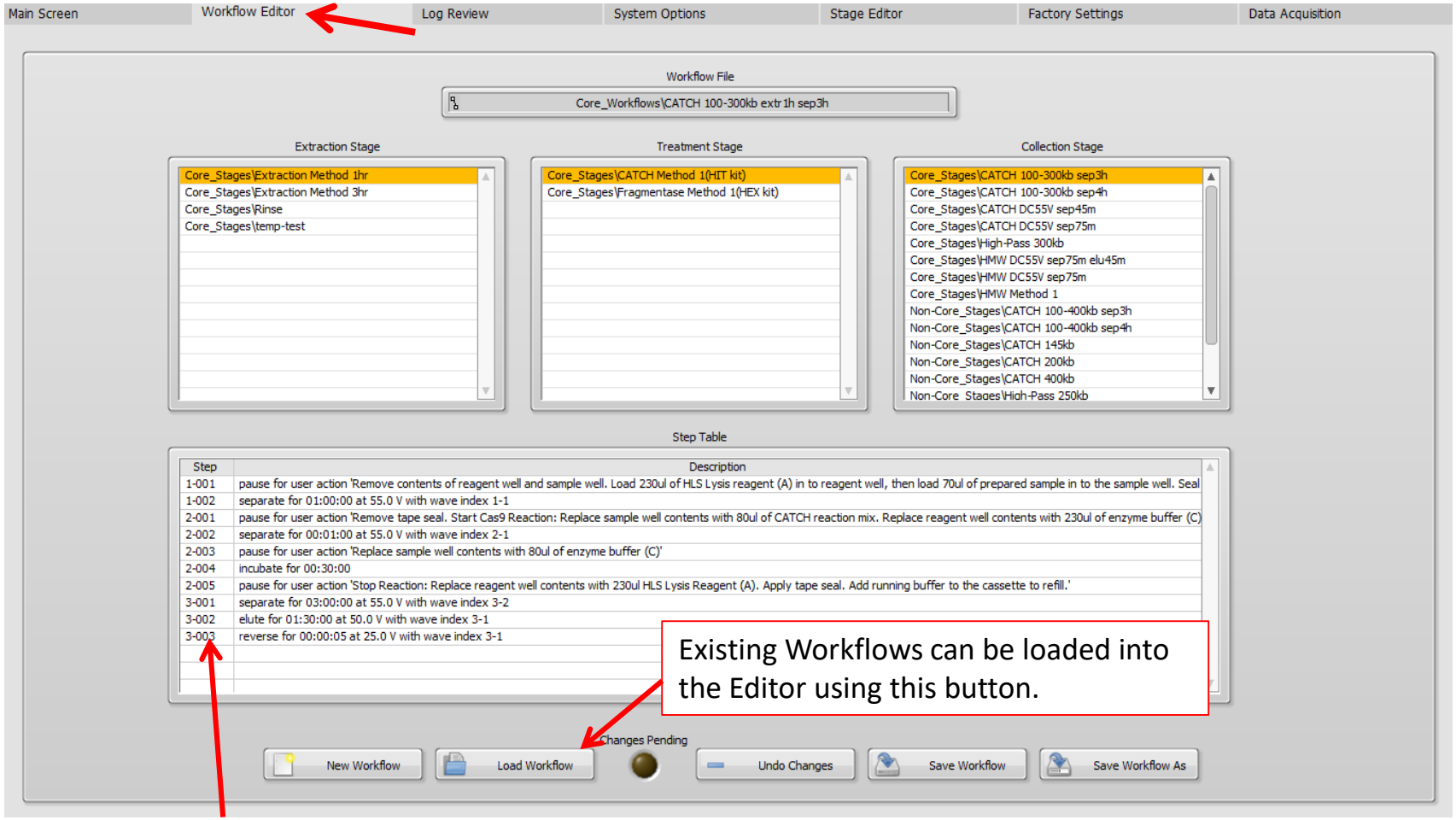
View of the main screen after selecting a CATCH workflow.

To view the stage structure of a particular HLS workflow, it is more convenient to load the workflow into the **Workflow Editor**, as shown on the next page.



HLS **Workflows** can be viewed, created, and edited in the **Workflow Editor**.

Once an existing workflow is loaded into the Editor, each component stage used by the workflow is highlighted in yellow.



Step numbering format is X-YYY, where X indicates stage number, and YYY indicates the step number in the X stage definition.

Notes on HLS-CATCH Extraction (Stage 1)

Extraction time considerations:
Sage has found that a variable amounts of non-specifically cleaved DNA (50kb to 200kb) is liberated from non-viable cells during the HLS extraction step. The 1 hour extraction time doesn't remove all of these non-specific fragments, and so they will contaminate the specific targets when using the 1 hr extraction time.

Current "Core" CATCH Workflows

CATCH 100-300kb extr1h sep3h.shflow
CATCH 100-300kb extr1h sep4h.shflow
CATCH 100-300kb extr3h sep3h.shflow
CATCH 100-300kb extr3h sep4h.shflow
CATCH DC55V extr1h sep45m.shflow
CATCH DC55V extr1h sep75m.shflow
CATCH DC55V extr3h sep45m.shflow
CATCH DC55V extr3h sep75m.shflow

This non-specific contamination is **actually beneficial** when library construction and sequencing is carried out with the 10x Genomics Chromium system. ***So the 1hr extraction workflows are recommended for 10x Genomics customers.***

For other sequencing platforms (PacBio and Oxford Nanopore), which employ less efficient library prep procedures, purer CATCH target products are needed, and the **3hr** extraction workflows are recommended.

Notes on HLS-CATCH Stage 3 Collection Stages

Sage R&D is actively developing new electrophoresis conditions for CATCH applications. Currently these efforts are focused on increasing the size range and resolution of the Stage 3 Collection electrophoresis step. Workflows that have been more thoroughly tested are released as "Core" workflows, while less tested workflows are released as "non-core".

Current "Core" CATCH Workflows

- CATCH 100-300kb extr1h sep3h.shflow
- CATCH 100-300kb extr1h sep4h.shflow
- CATCH 100-300kb extr3h sep3h.shflow
- CATCH 100-300kb extr3h sep4h.shflow
- CATCH DC55V extr1h sep45m.shflow
- CATCH DC55V extr1h sep75m.shflow
- CATCH DC55V extr3h sep45m.shflow
- CATCH DC55V extr3h sep75m.shflow

Workflow names for HLS-CATCH follow the convention:

CATCH <collection electrophoresis waveform name> extrXX(h or m) sepYY(h or m).

where XX denotes extraction time, and YY indicates collection stage time, and "h", "m" indicate time units of hours or minutes, respectively.

Notes on HLS-CATCH strategies for size selection and Stage 3 Collection Stages (cont.)

Target size considerations:

The “100-300kb” waveform has been successfully tested for 200kb CATCH targets with 3 hour separations, and for 400kb targets with 4 hour separations (see slide 45). It has also been tested for 100kb CATCH targets with the 3 hour separation time.

The “DC55V” waveform has been used successfully for CATCH targets up to 16 kb in size with both 45 and 75 minute separation times. With the 75 minute separation time, the CATCH target range should extend up to 30kb.

Current “Core” CATCH Workflows

- CATCH 100-300kb extr1h sep3h.shflow
- CATCH 100-300kb extr1h sep4h.shflow
- CATCH 100-300kb extr3h sep3h.shflow
- CATCH 100-300kb extr3h sep4h.shflow
- CATCH DC55V extr1h sep45m.shflow
- CATCH DC55V extr1h sep75m.shflow
- CATCH DC55V extr3h sep45m.shflow
- CATCH DC55V extr3h sep75m.shflow

Cas9 enzyme assembly for HLS-CATCH

Overview of Cas9 enzyme assembly process

The following protocol assumes use of IDT ALT-R™ two-part synthetic guide RNAs, and S. pyogenes wild-type Cas9 enzyme from New England Biolabs. We have had consistently good results with these materials.

This protocol assembles the active Cas9 reaction mix in three steps.

1) The two-part guide RNAs are annealed by heating to 95C and cooling to room temperature.
2) The annealed guide RNAs are mixed with the Cas9 enzyme and incubated briefly at 37C.
3) The final assembled enzyme is diluted to the correct volume (80 ul) for loading into the HLS sample well.

Step 1. Annealing the two-part IDT ALT-R™ guide RNAs

Dissolve each of the crRNAs and tracrRNAs to be used at **100uM** concentration in IDT Duplexing Buffer. The correct volume for resuspension in microliters will be 10 times the number of nanomoles of RNA in the tube supplied by IDT.

To a 200 ul PCR tube add:

1) 15.4 ul IDT duplexing buffer.

2) 4 ul of a pooled mixture of all crRNAs used in the experiment. To make the pool, combine equal volumes of each stock crRNA solution. All crRNAs stock solutions should be at 100 uM, in IDT Duplexing Buffer.

(For instance, if two crRNAs are to be used, add 2 ul of each crRNA solution. If four crRNAs are to be used, add 1 ul of each crRNA solution. For more crRNAs, mix equal volumes of each crRNA (using some convenient volume), and then add 4 ul of the resulting crRNA mixture to the PCR tube. The goal here is to saturate the tracrRNA with a 1.5-fold molar excess of total crRNA.)

Mix the duplex buffer and crRNAs thoroughly by vortexing or pipetting, and spin down briefly.

3) Add 2.6 ul of tracrRNA (100 uM).

Mix thoroughly and spin down briefly. (Vol. =22 ul. Conc. after this step = 11.8 uM tracrRNA, 18 uM total pooled crRNA.)

4) Heat the 20 ul mixture for 5 minutes at 95C in a thermal cycler with a heated lid, then remove the tube from the cycler and allow to cool on the lab bench at room temp for 3-5 minutes. Centrifuge for 30-60 seconds to collect any condensation.

Total volume of the annealed RNA will be **approximately 22 ul**.

(We have found that annealed guide RNAs can be stored for at least 3 weeks at -20C with no loss of activity.)

Step 2. Assemble annealed guide RNAs and Cas9

Add in order:

1) 10 ul Sage HLS 4X Enzyme Buffer.

2) 22 ul annealed IDT guide RNAs (from Step 1). Mix thoroughly by vortexing or pipetting and spin down briefly.

3) 8 ul NEB wild-type S. pyogenes Cas9 enzyme (20 uM). Mix thoroughly by gentle pipetting of entire mixture (40ul). Conc. after this dilution step: 4 uM Cas9 enzyme, 6.5 uM annealed tracrRNA.

4) Incubate mixture at 37C for 10 minutes to assemble enzyme and gRNAs. Proceed to dilution (step 3), or store on ice until ready.

Step 3. Dilute to loading concentration with HLS Enzyme Buffer.

Dilute assembled Cas9 mixture from Step 2 (40ul), with 40 ul of HLS Enzyme Buffer. Mix thoroughly by gentle pipetting of the entire mixture (80 ul). (Final conc: 2 uM Cas9 assembled with 3.25 uM pool of annealed two-part gRNAs)

At this point the Cas9 mixture is ready to load in the HLS sample well. The mixture can be stored on ice, or at 4C, for several hours.

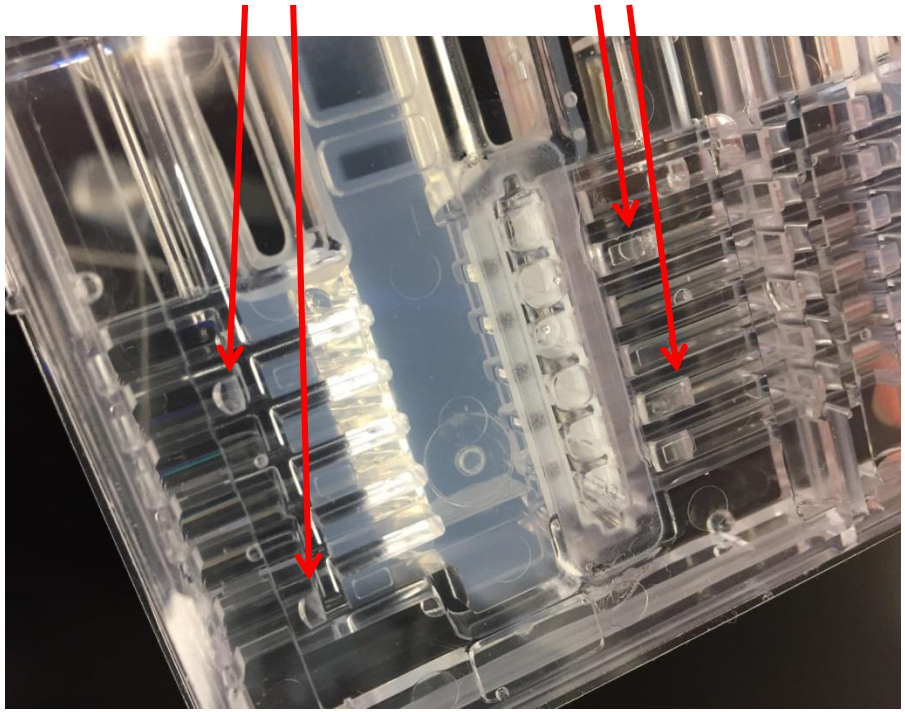
(According to some of our customers, the assembled ready-to-load Cas9 mixtures is stable for at least 48 hours at 4C. We will be testing 4C storage of assembled enzyme and will report when we have data.)

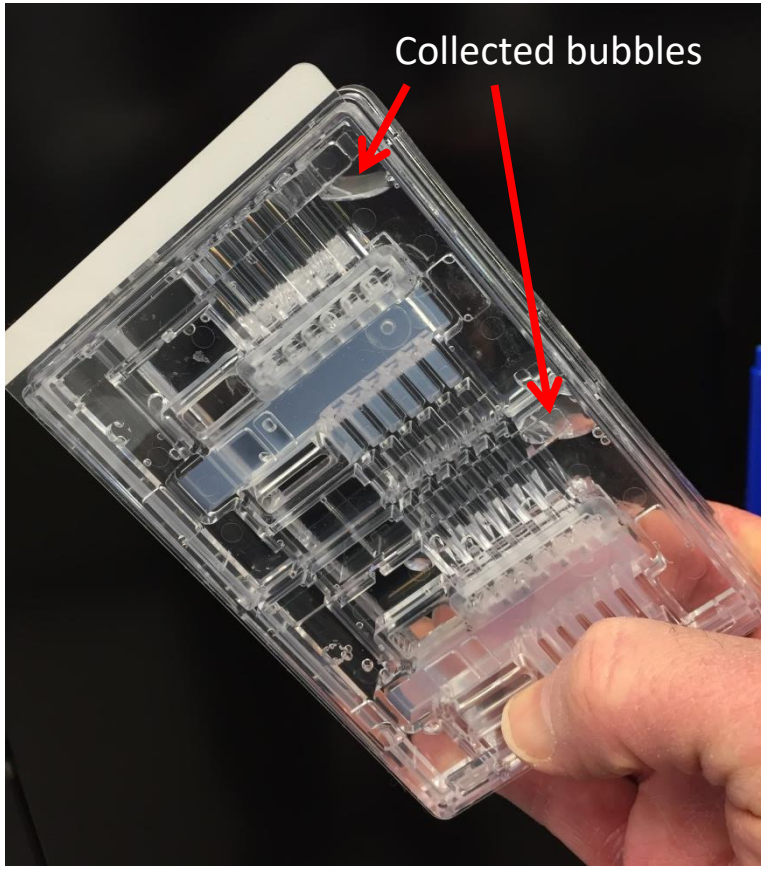
HLS Cassette Preparation

HLS Cassette Preparation- Collecting trapped bubbles.

Hold the sealed cassette with its surface plane vertical to the ground. Keeping the cassette surface vertical, slowly rotate the sealed cassette 360 degrees while tapping the top surface of cassette to remove all trapped air bubbles from the elution channels. Collect all the bubbles into one end of the reservoirs (either end is OK). The collected bubbles will escape after the cassette is opened and topped up with buffer before run.

Trapped air bubbles that interfere with elution





SageHLS – Cassette Preparation: Remove tape, Top-up buffer, Perform current check.

1. Grasp tabbed edge of sealing tape with one hand, hold cassette firmly in nest with other hand.

2. With a slow side-to-side movement, slowly peel off sealing tape. Go slow. Avoid contact with buffer in electrode port openings.

3. Completely empty and refill all the elution modules in each lane to ensure no bubbles are trapped inside. (Keep pipette tips vertical to avoid scraping membranes on each side of the elution module.) The elution modules hold approximately 80-85 ul of buffer. Refill each module with 80 ul of Running Buffer.

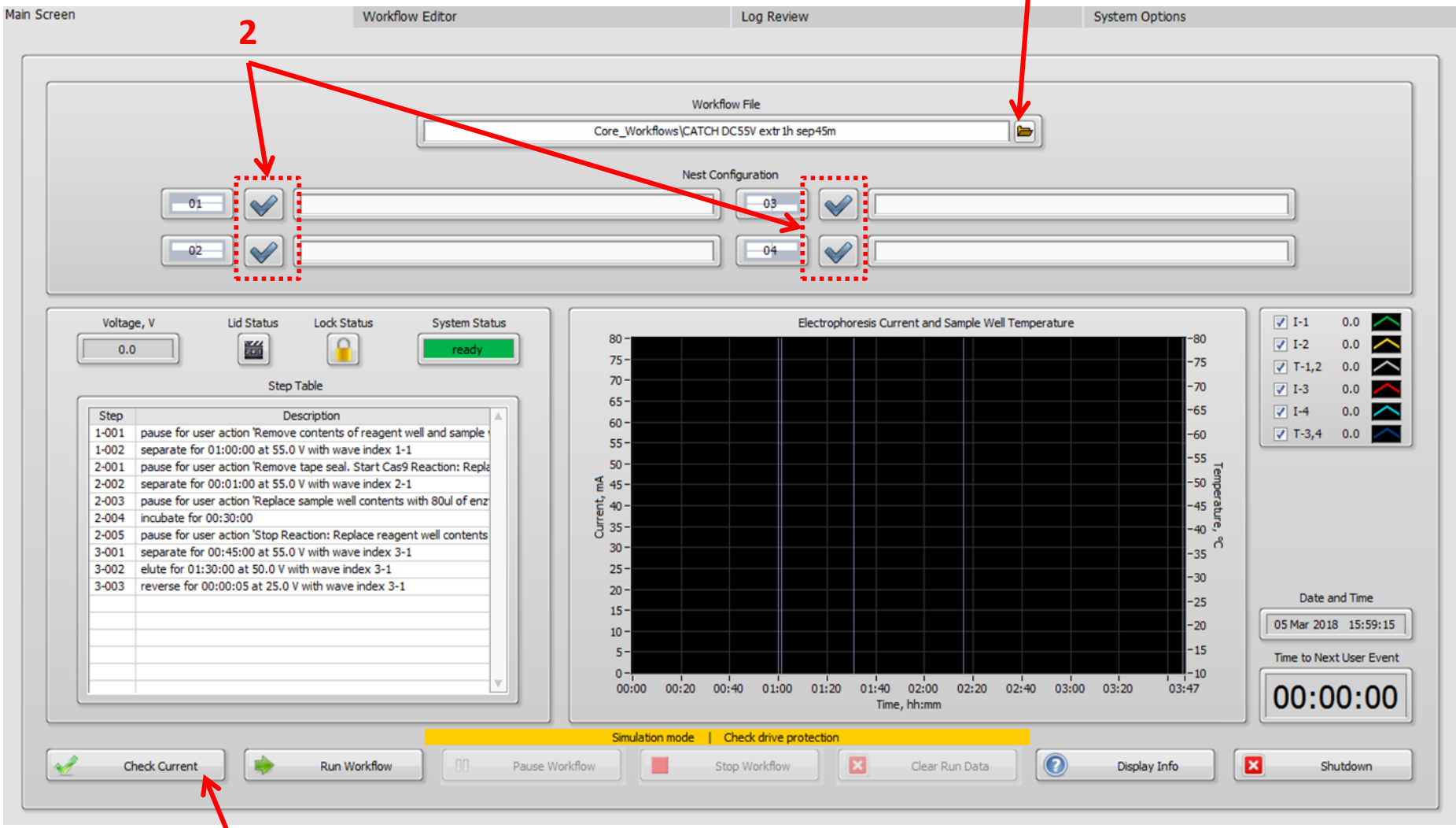
4. Top up the volume of the Reagent and Sample wells. These wells should have a slight concave meniscus when properly filled. The maximum volumes of the wells are approximately 270 and 85 ul, respectively.

5. Completely fill the electrode buffer chambers with Running Buffer. The electrode channels at the ends and sides of the cassette should be filled level with the top surface of the cassette.

6. Close lid, perform current check on lanes to be used for the experiment. (See next page for instructions on performing current check).

Current check procedure (brief version)

1. Select workflow file.
2. Choose lanes to be used.
3. Close lid. Click “Check Current”

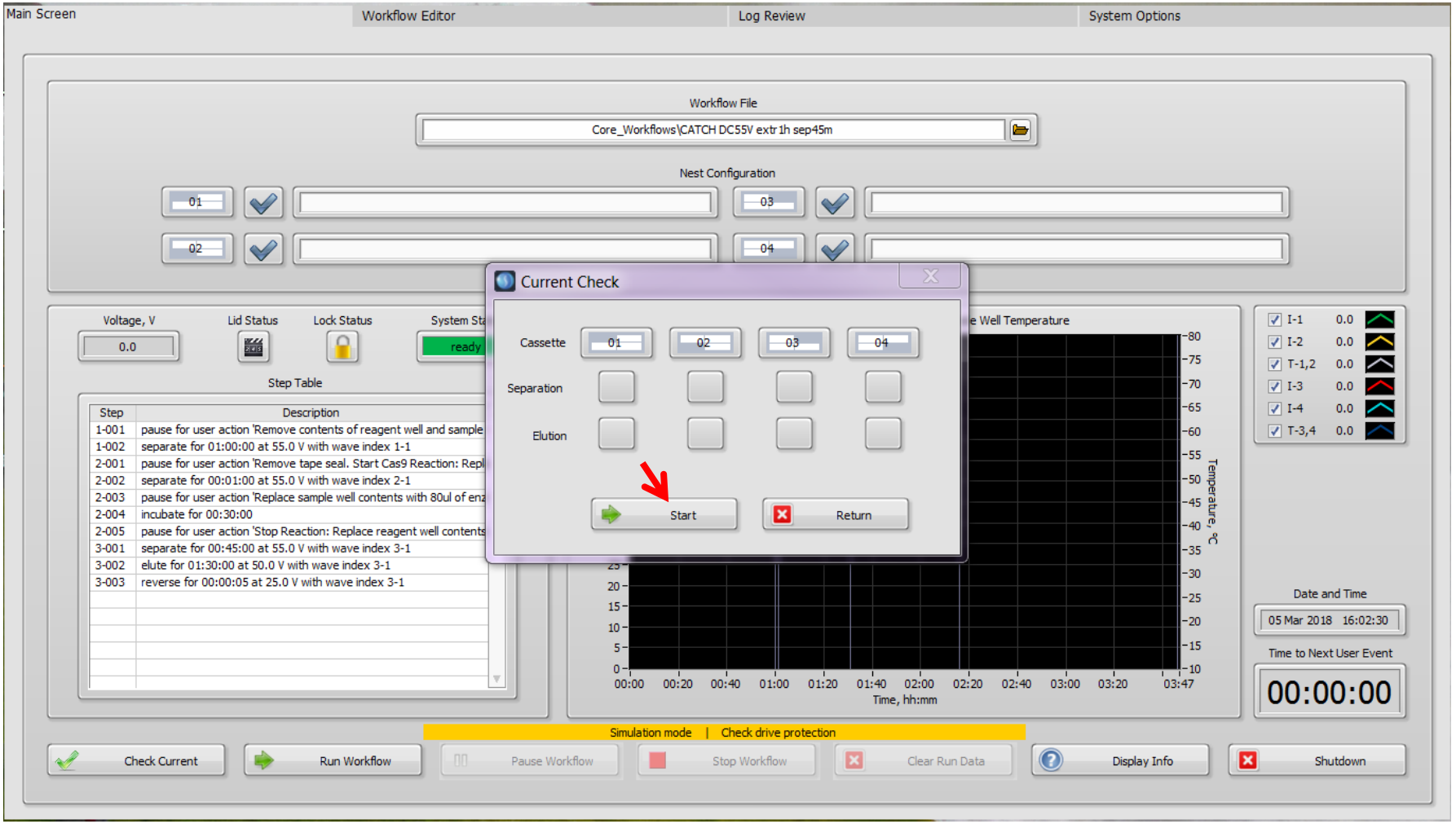


Current Check Procedure (cont.)

Pop-up “Current Check” window appears.

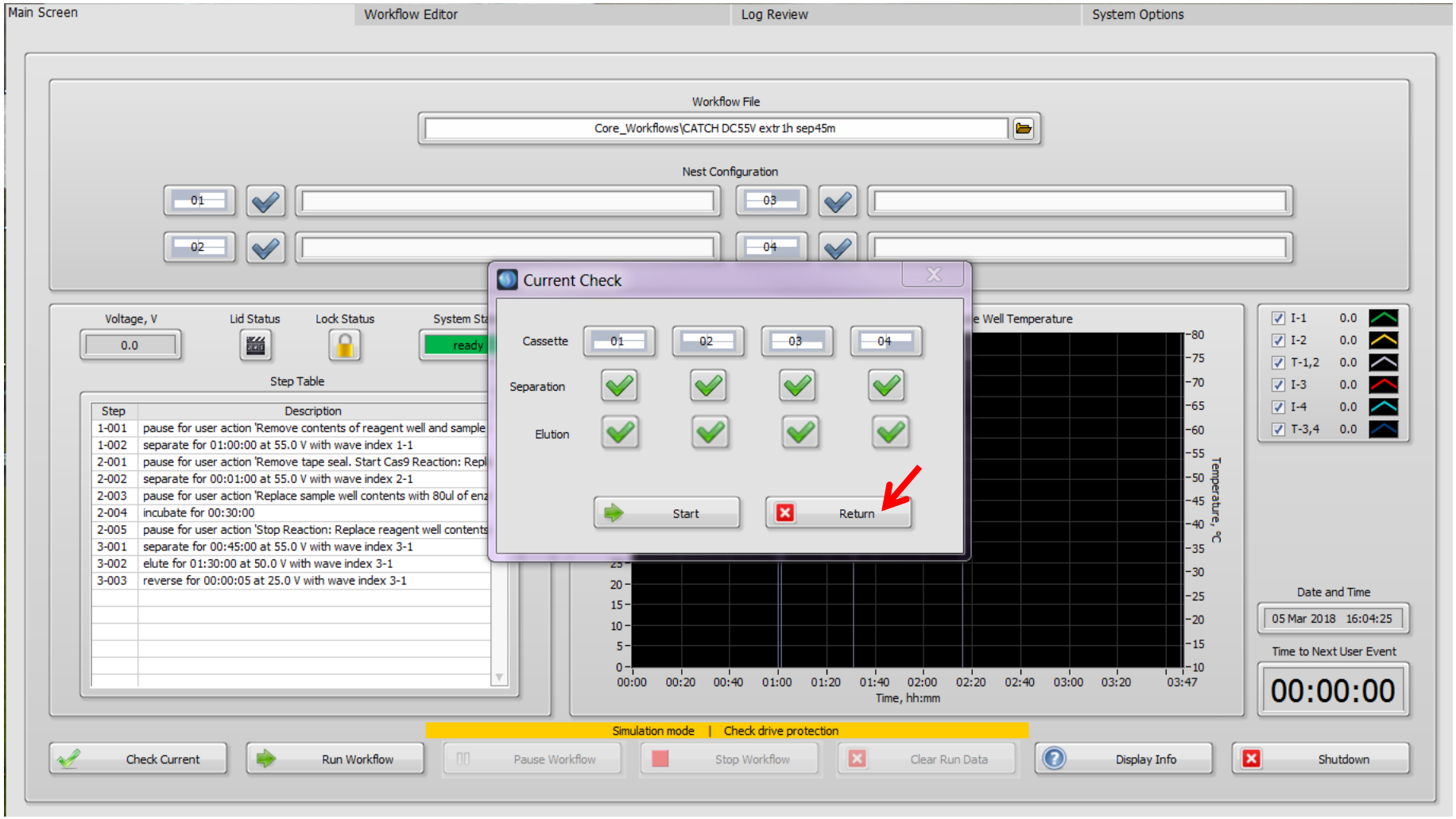
Click on “Start”.

Lid lock will click, and elevator will be heard.



Current Check Procedure (cont.)

Test takes a couple of minutes, first testing Separation electrodes, then Elution electrodes.
After successful current test all boxes will be filled with green checkmarks.
Hit “Return” in popup to get back to “Main”.

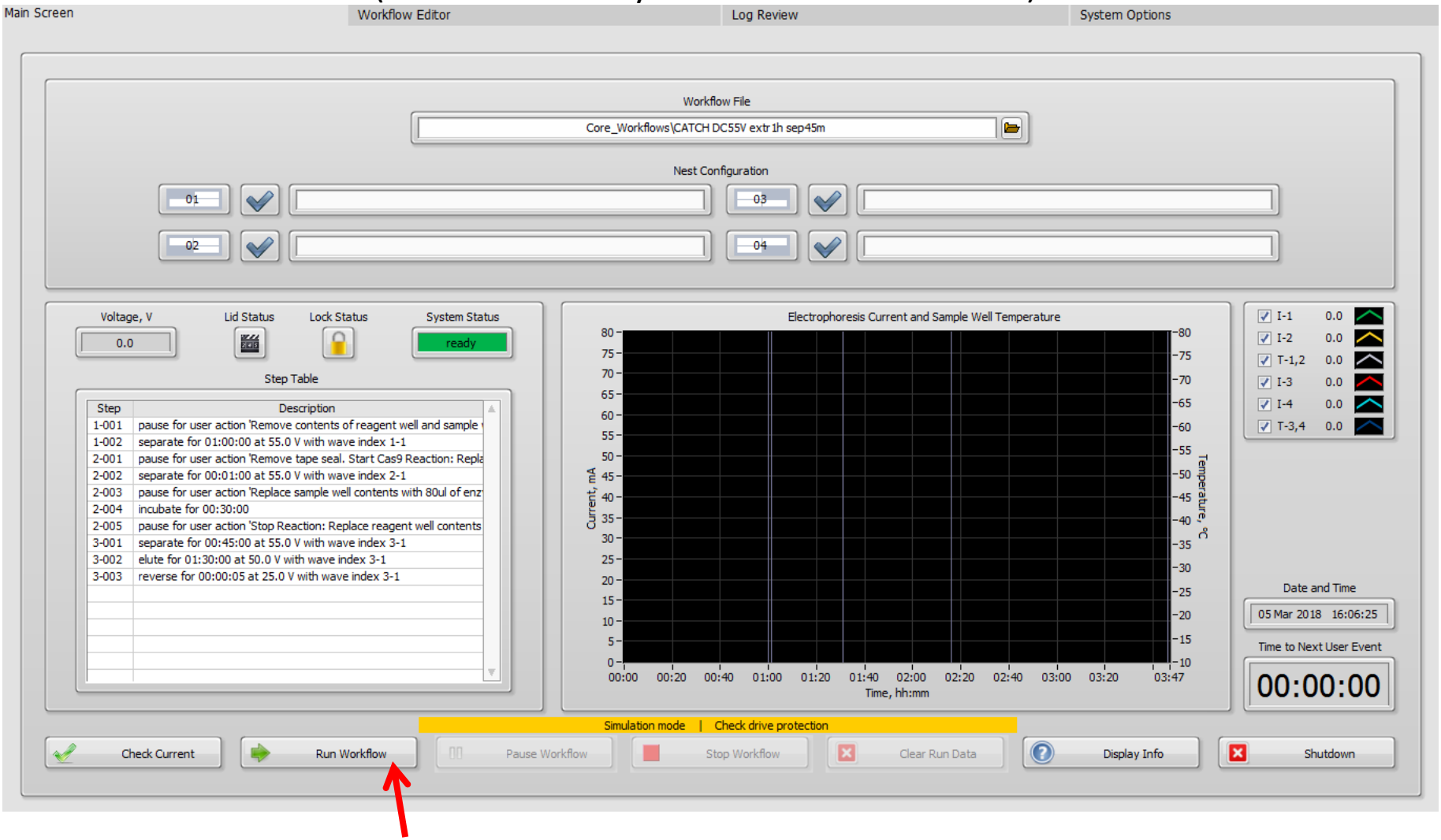


HLS-CATCH Workflow and User Actions

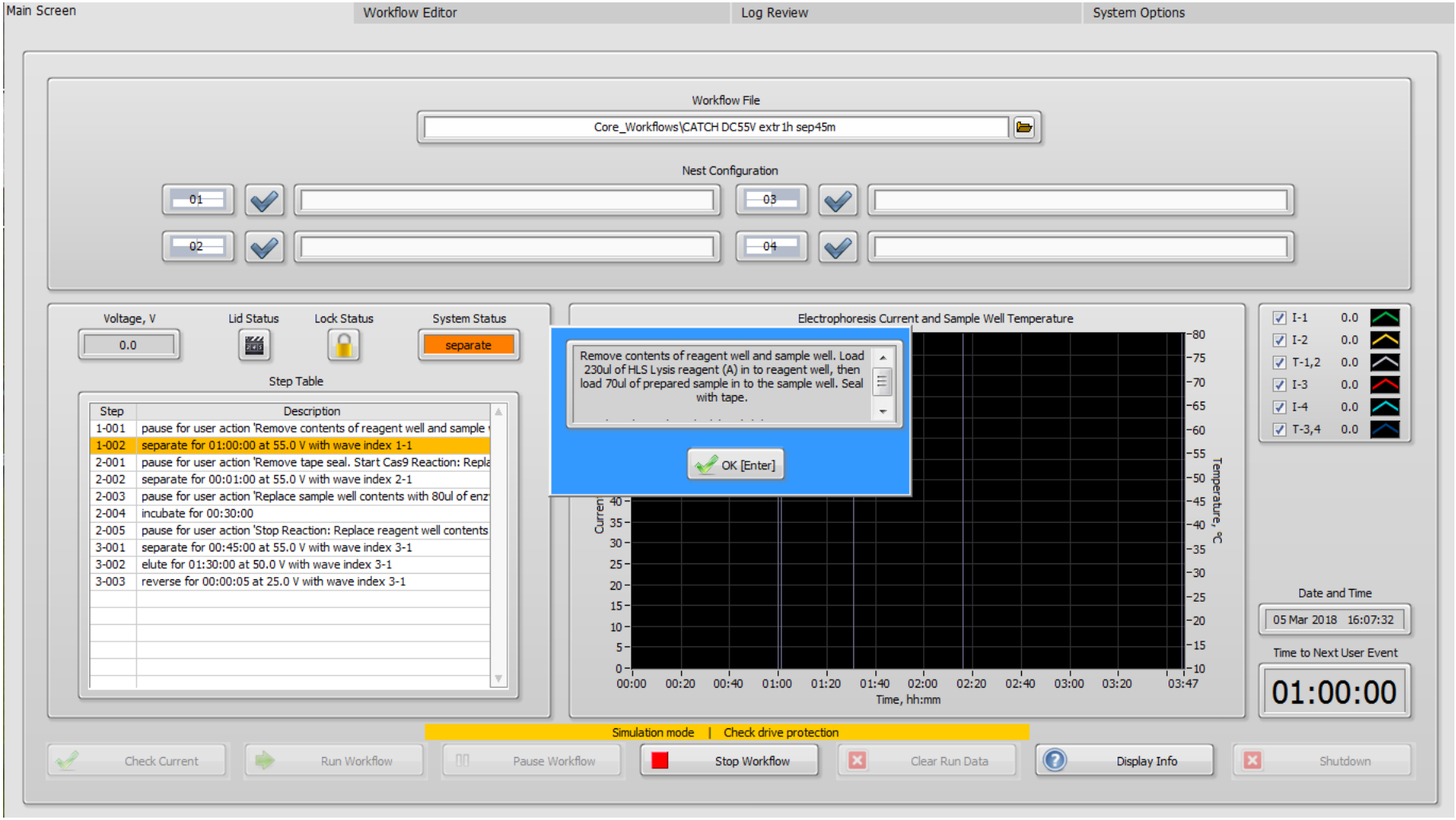
Main screen: Ready to start Stage 1- Extraction

Click “Run Workflow” command.

(Command is only active after lid is closed.)



Popup window prompts user to replace sample and reagent well contents with sample and lysis reagent. See following slides for detailed instructions.



Initiating Stage 1- Extraction

User actions **after** current-check, at start-of-run pop-up prompt.

1) Empty all sample and reagent wells. Take caution not to pierce agarose at bottom of wells.

2) Load samples in all lanes. Always use 70ul sample loading volume. (Sample wells will not be completely full.)

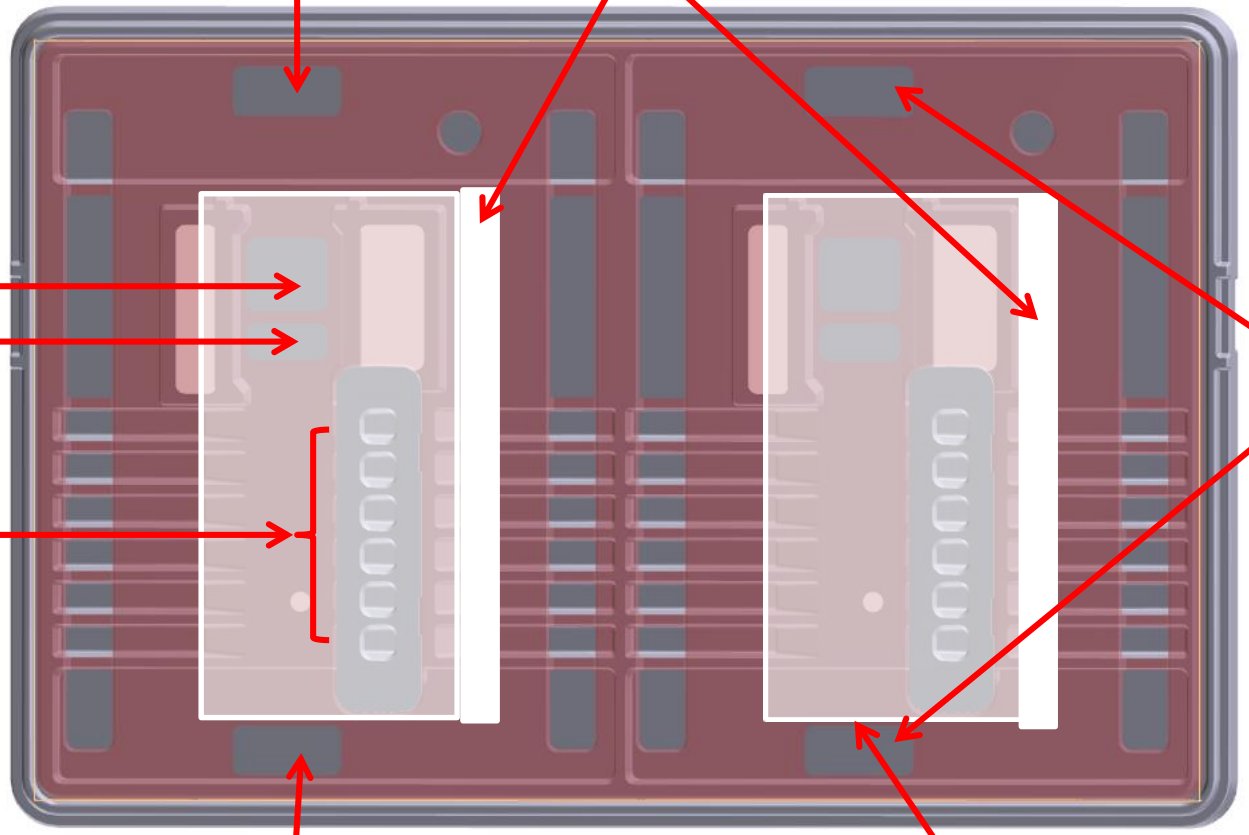
3) Fill Reagent Wells with HLS Lysis Buffer. Fill, but do not overfill! Leave a concave meniscus to prevent contact with sealing tape in next step. Approximate volume needed will be 220-230 ul.

4) Seal reagent, sample, and elution ports with supplied tape. Press tape firmly around edges of ports (see next slides for taping details), and close lid.

5) Click on “OK”.

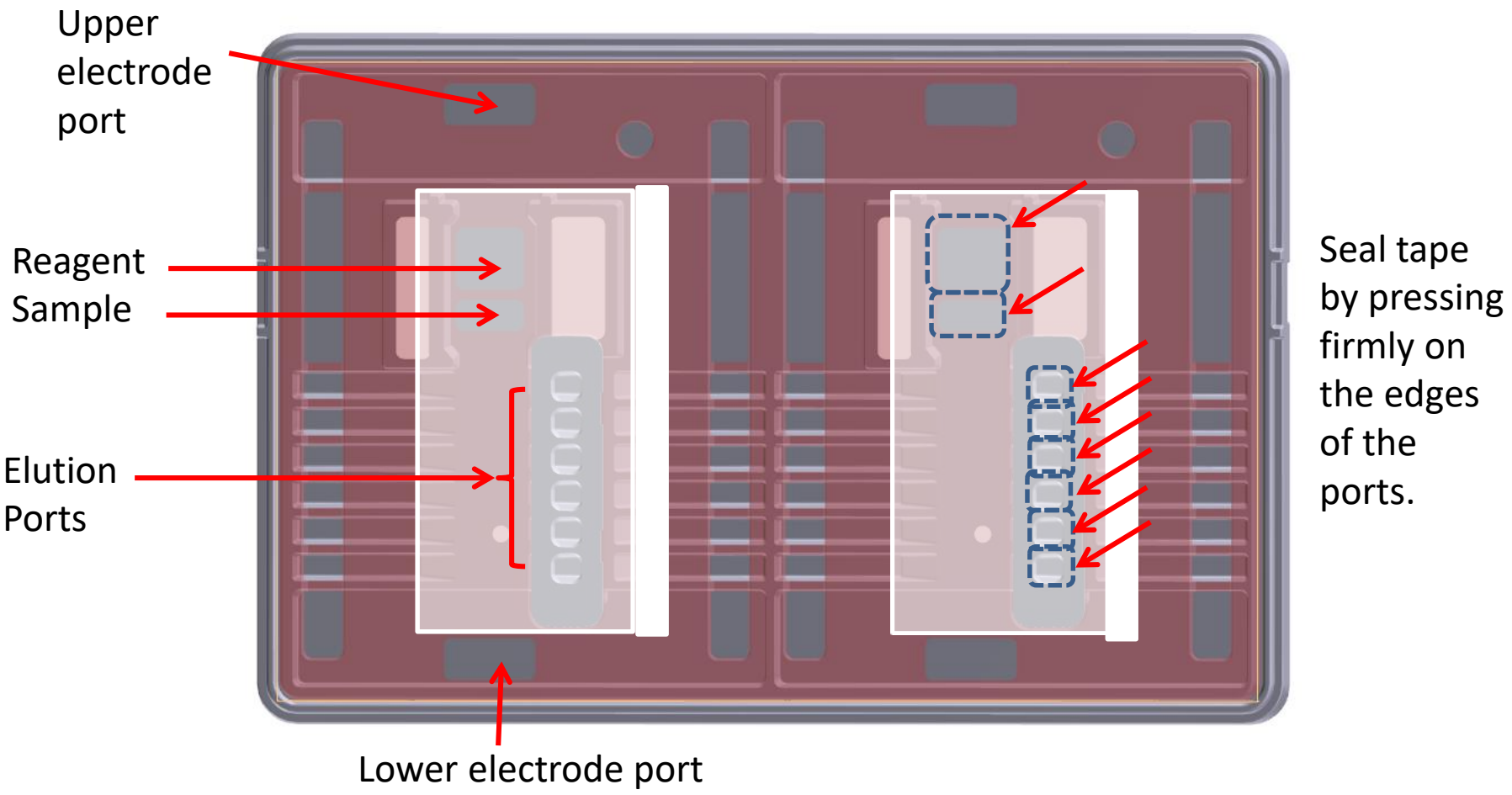
Placement of sealing tape after cassette loading

Be sure that all Reagent, Sample, and Elution ports are completely covered.



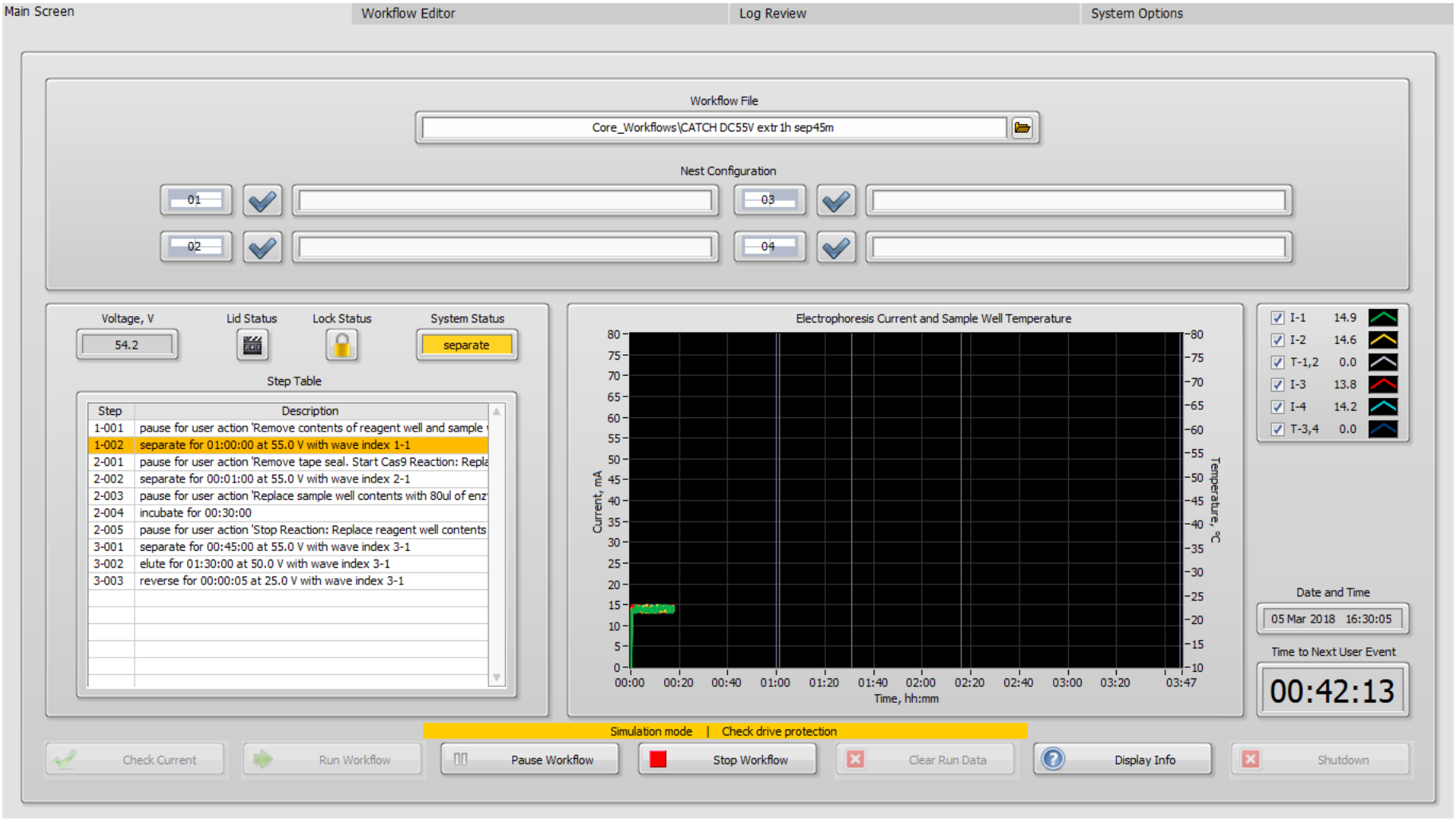
Seal tape securely around port borders

The tape is pressure-sensitive. After placing tape on cassette, **press tape tightly to the cassette surface at the edges of the reagent, sample, and elution ports** using a hard, smooth, round object like the back end of a lab marker, or the back side of a curved spatula. Take care **not to press on tape directly over open areas of the ports**, which could force liquid out under the tape.



Main screen during Stage 1 - Extraction (after 18 min)

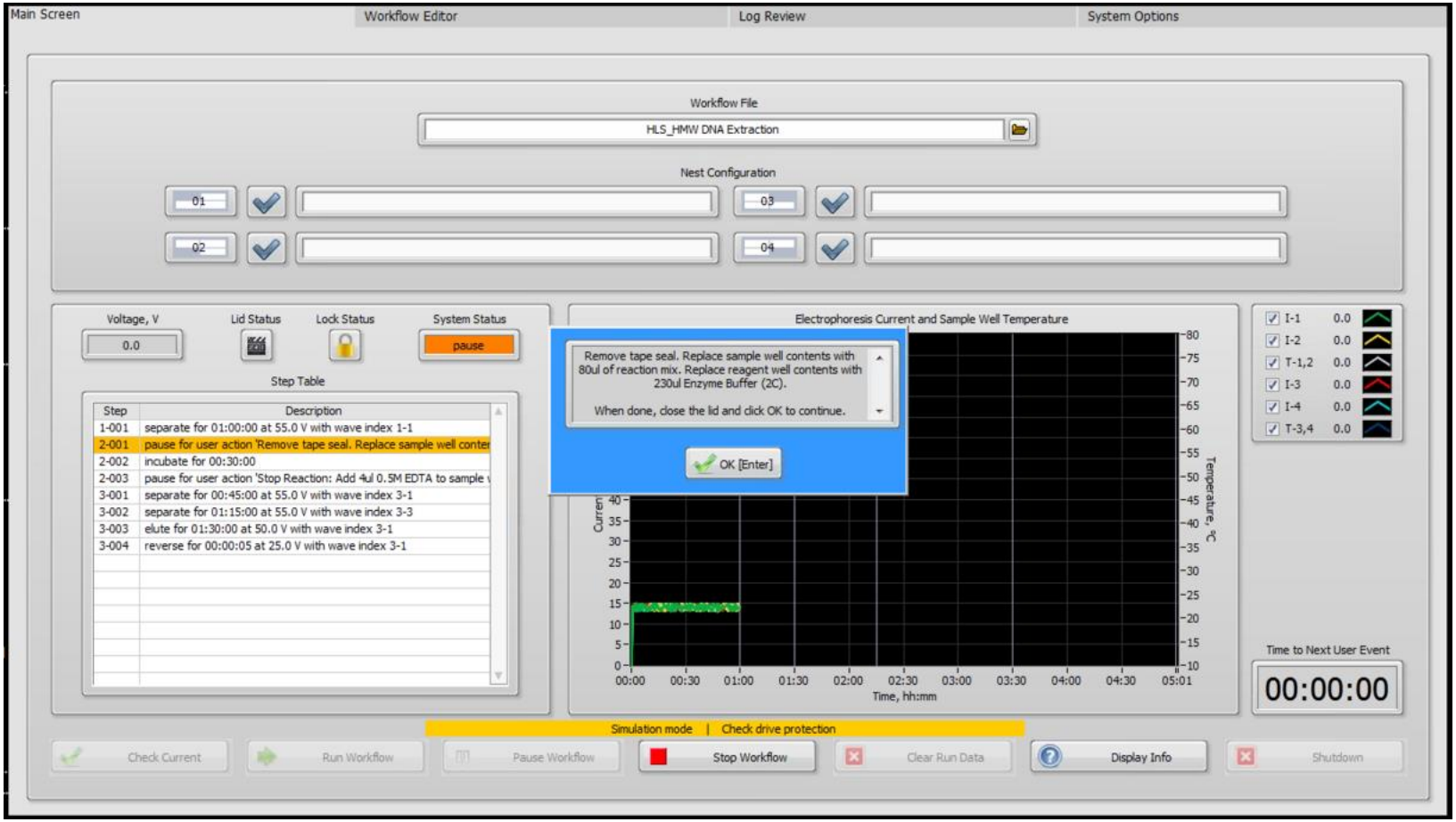
Current should be similar for all lanes, and in the range of 10-15 mA.



End of Stage I - Extraction

Prompts appear in popup window.

See next slide for details on completion of stage 1 and initiation of Stage 2.



Completion of Stage 1 Extraction

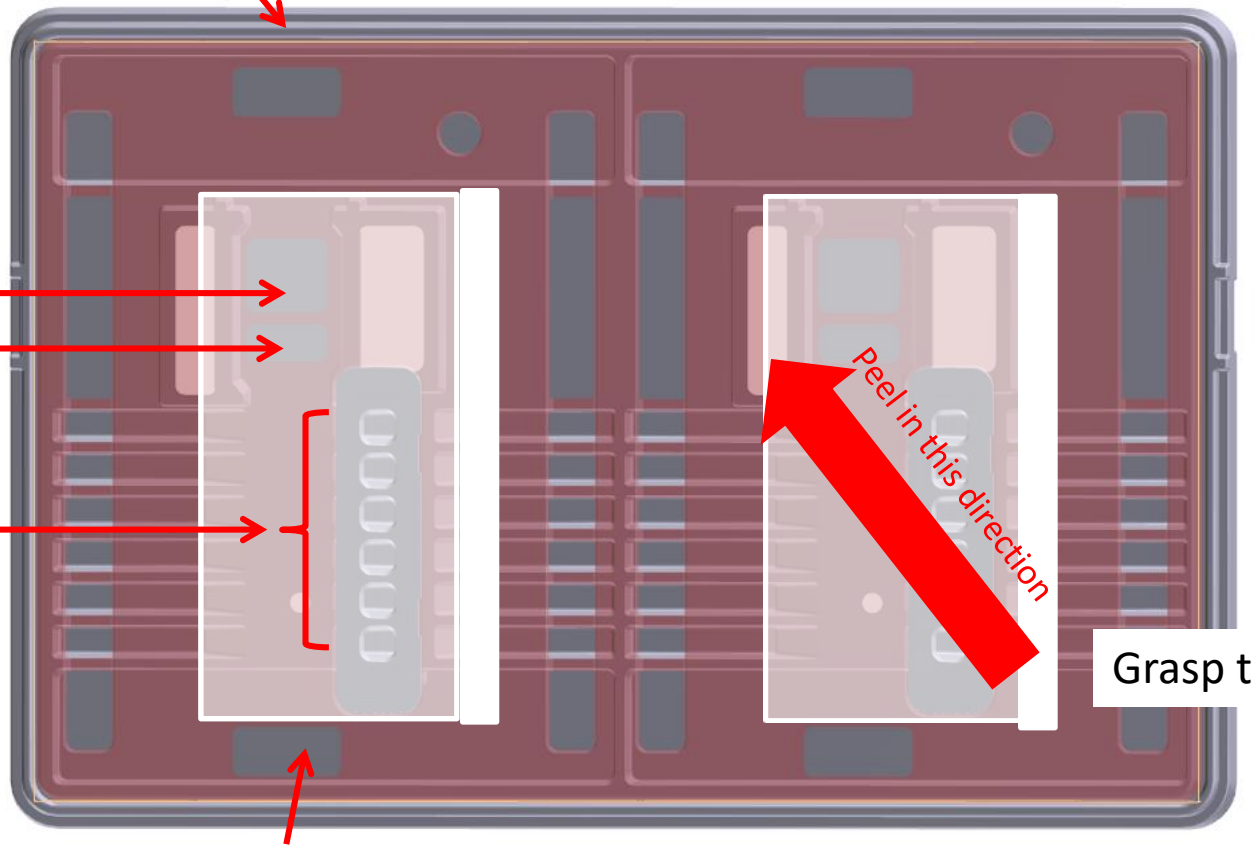
Open lid. Remove 2-5 ml of foamy, SDS-rich electrophoresis buffer from the lower separation electrode port of each cassette lane, and discard it.

Remove port sealing tapes by pulling the tabs in a diagonal direction as illustrated on the next slide. (Removing the tape in this manner reduces the risk of introducing liquids from the Reagent or Sample well ports into the elution modules.)

Refill the electrode buffer chamber with fresh electrophoresis buffer so that the buffer level is even with the top surface of the cassette.

Completion of HLS Stage 1: Removing tape

Grasp tab on elution module side, peel slowly in diagonal direction, **away from elution modules**. Peeling on diagonal prevents cross-contamination of elution modules.



HLS-CATCH Stage 2 (treatment) Overview

After loading the Cas9 reaction mixture and clicking “OK” on the popup reagent addition prompt, the instrument performs a short electrophoresis pulse (1 minute) to “inject” the Cas9 complexes into the sample gel wall where the DNA is immobilized.

Following the 1 minute injection, the user empties the sample well and refills with HLS Enzyme buffer (without Cas9) to ensure that the Mg^{++} concentration remains relatively constant.

HLS-CATCH Initiation of Stage 2: Cas9 digestion

Remove and discard contents of **Sample well** and **Reagent well**.

Add 80 ul of Cas9 reaction mixture to the **Sample well**.

Fill **Reagent well** with HLS Enzyme Buffer (without enzyme); approximately 230 ul.

Close lid (do **not** retape the sample/elution ports at this step).

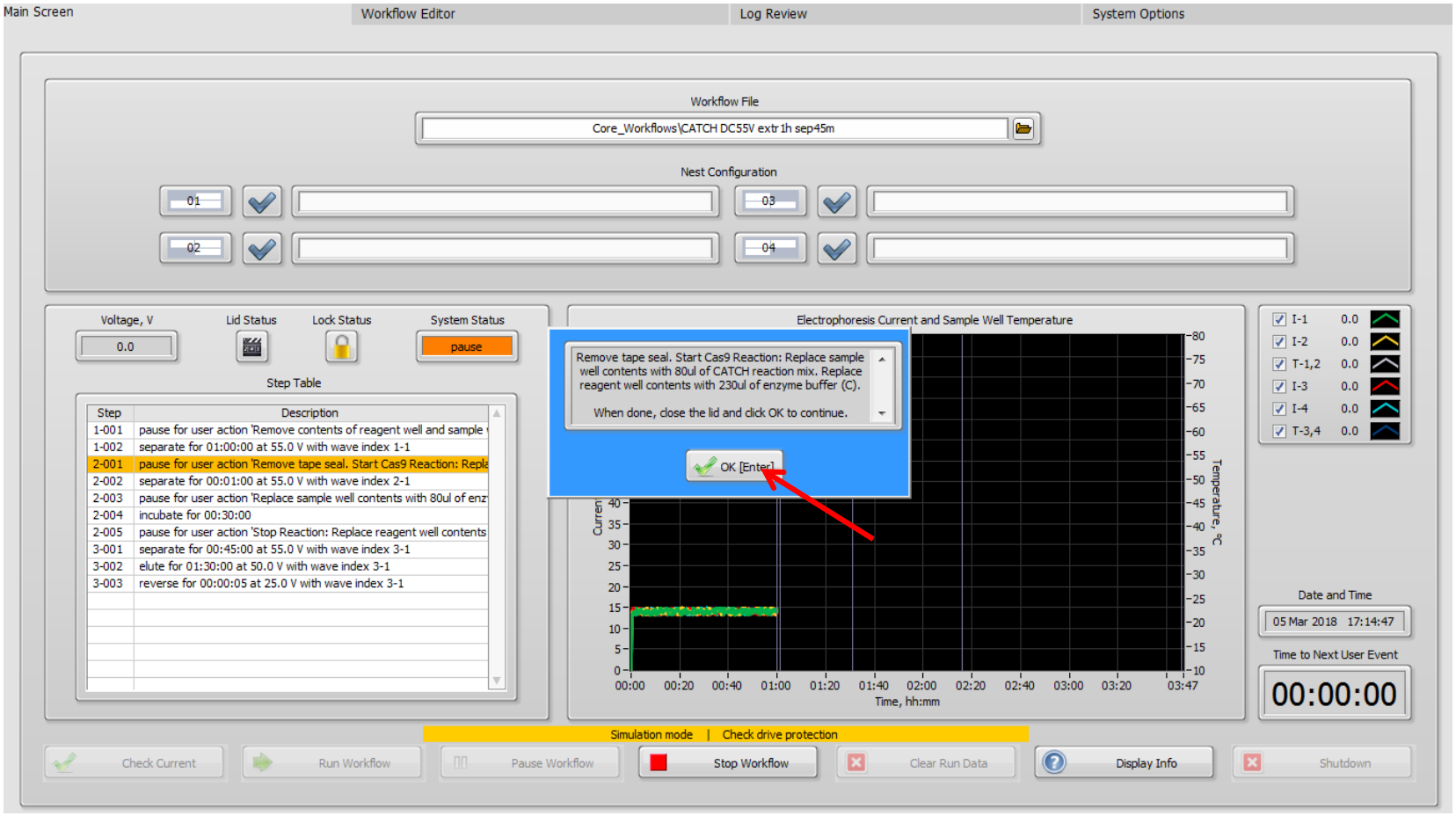
Click “OK” on the popup user prompt to initiate the 1 minute electrophoretic injection.

After injection, replace sample well contents with 80 ul of fresh HLS Enzyme buffer (without Cas9 enzyme).

Close lid (do not retape cassette), and click OK on popup prompt to start Cas9 digestion timer.

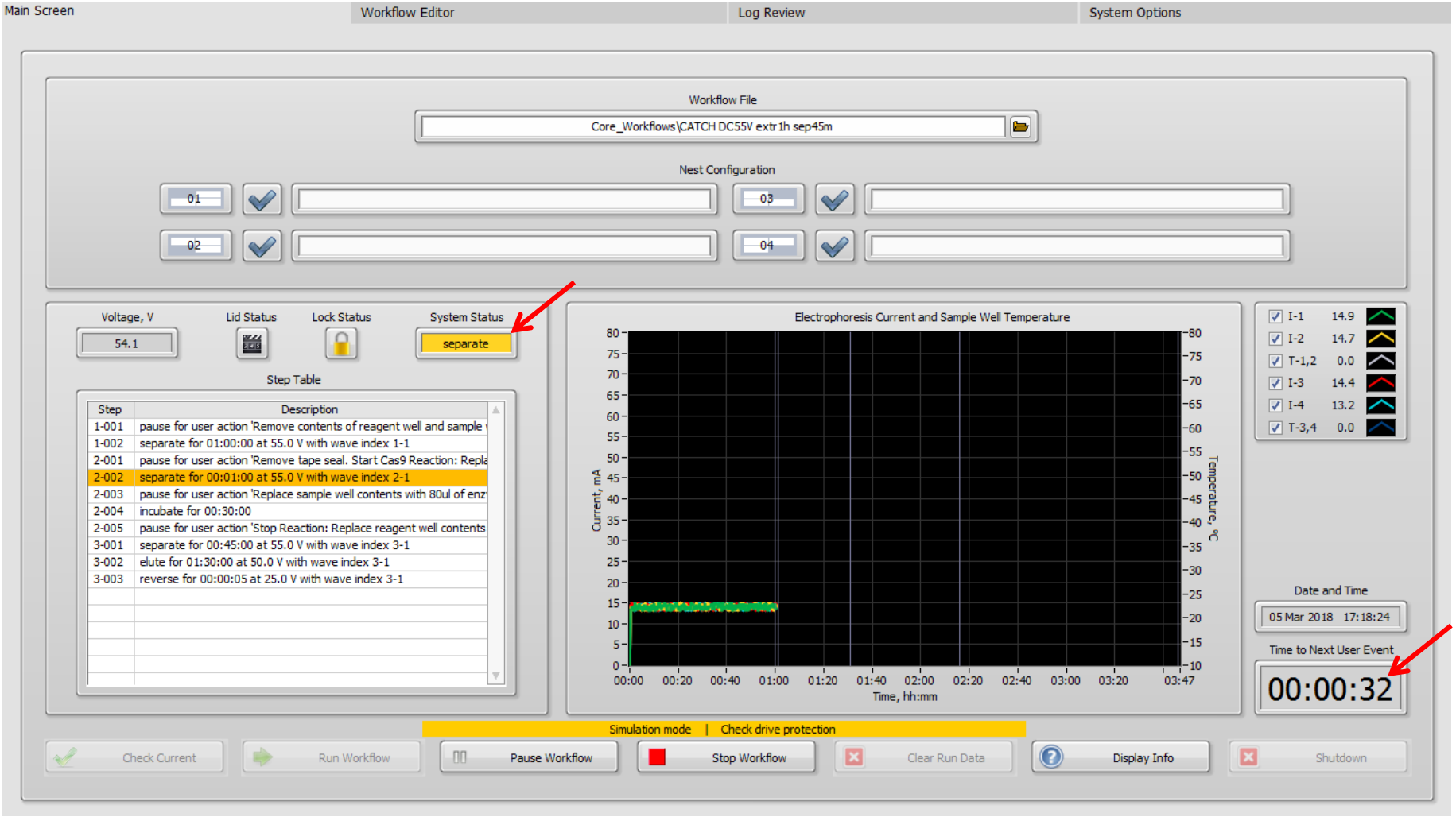
Screen at end of Stage 1/Beginning Stage 2

Popup prompt shows request to replace Sample and Reagent well contents as discussed on previous slide. After these replacements, do not retape. Close lid. Click OK. (Lid lock will click.)



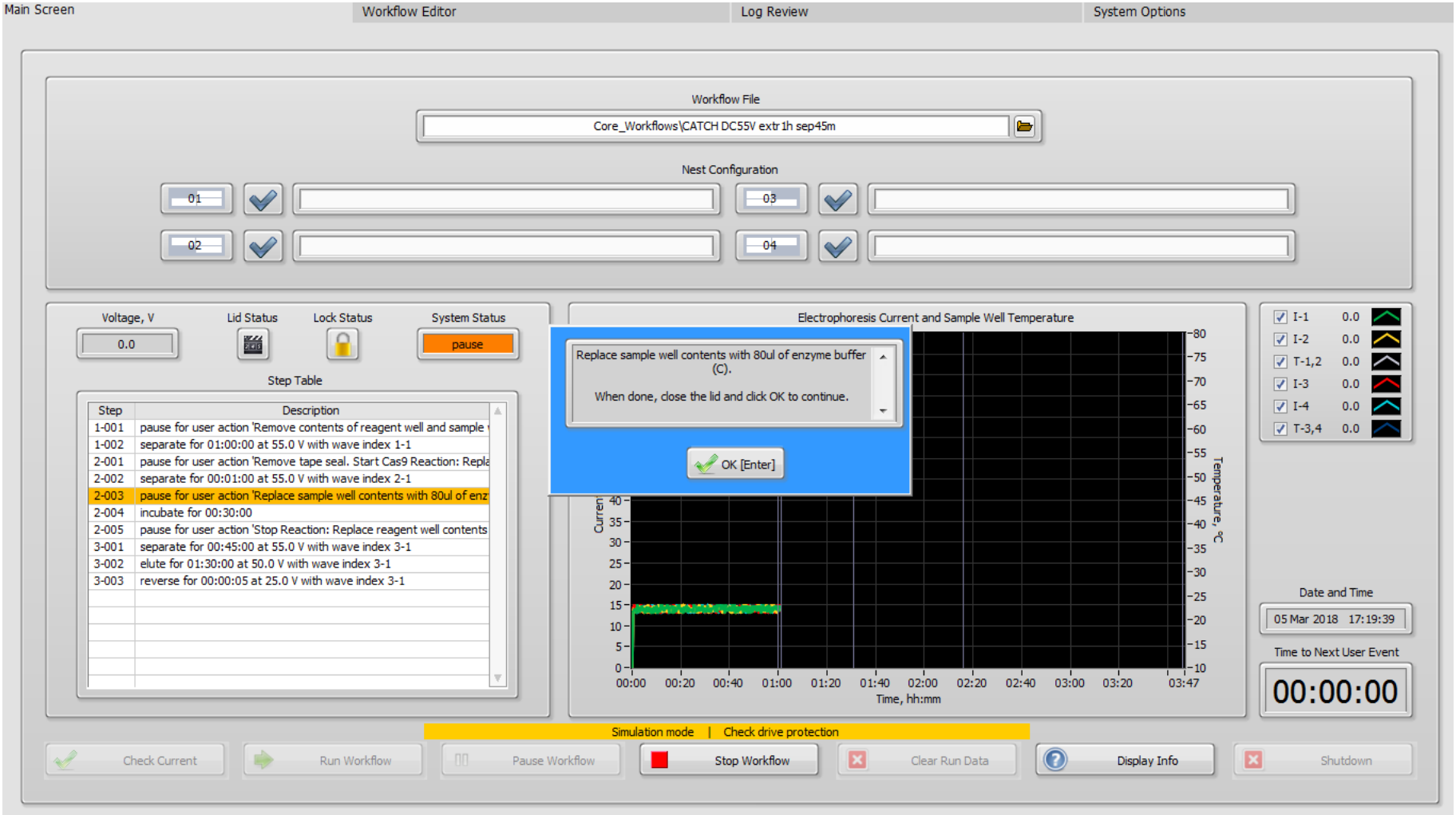
Screen after reagent addition at start of Stage 2.

Instrument performs **1 minute** of electrophoresis to inject Cas9 into the sample wall where the HMW DNA is immobilized.



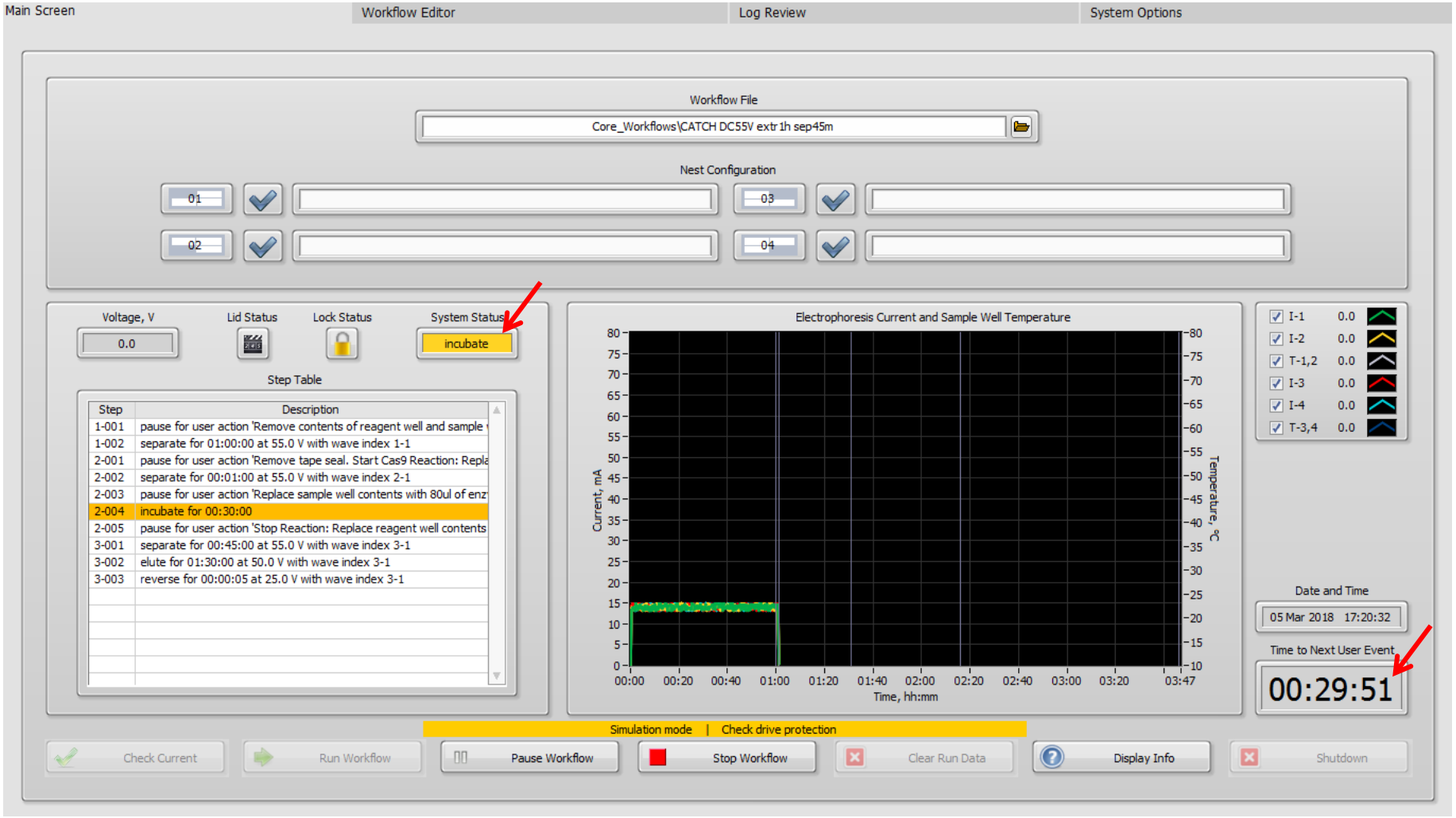
Screen after electrophoretic injection of Cas9

User is prompted to refill **Sample well** with 80ul of HLS Enzyme buffer (without Cas9).
Do not retape cassette. Close lid. Click OK. (Lid lock will click.)



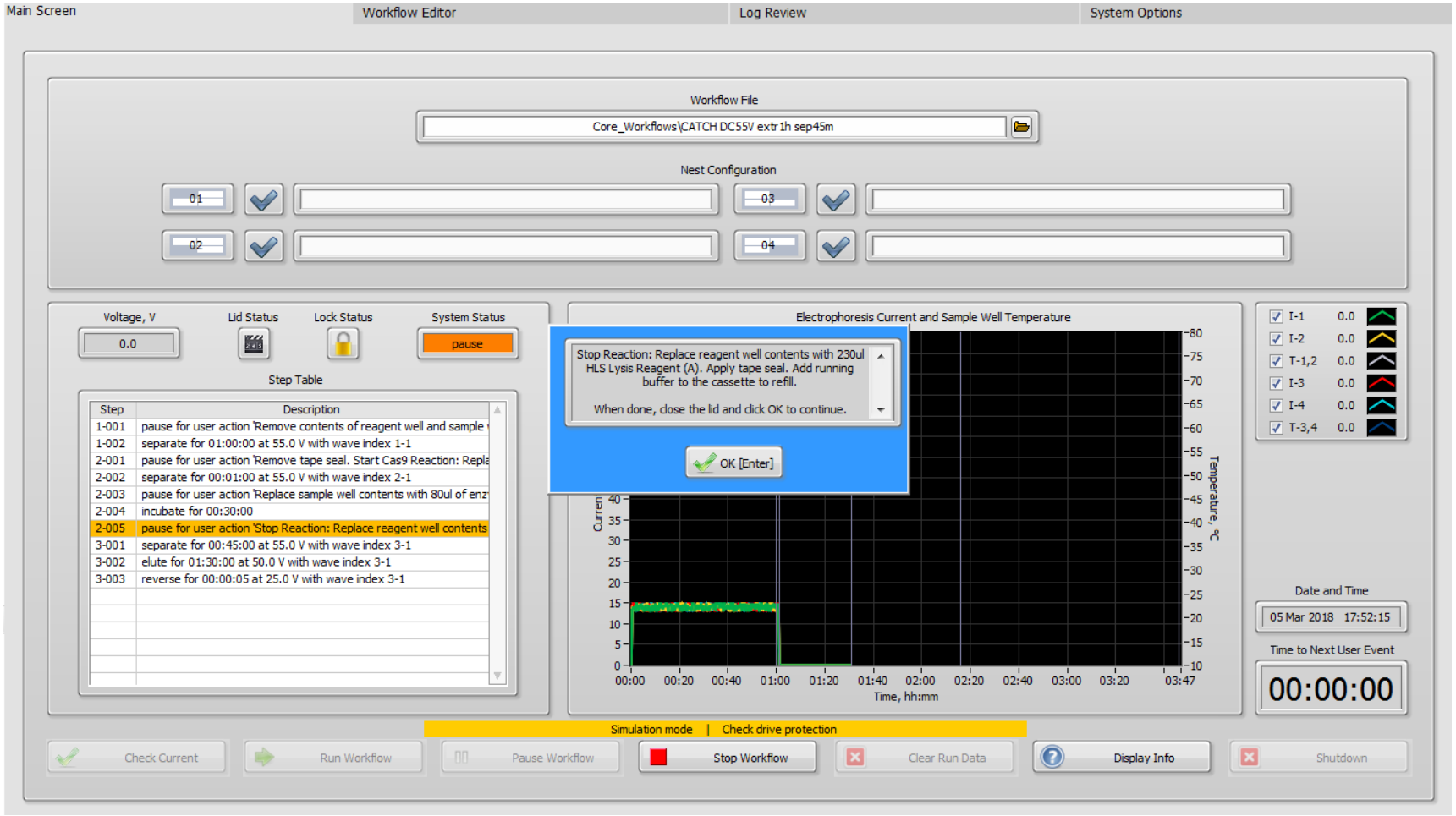
Screen after refilling Sample well with 80 ul of Enzyme buffer

Cas9 digestion takes 30 minutes.



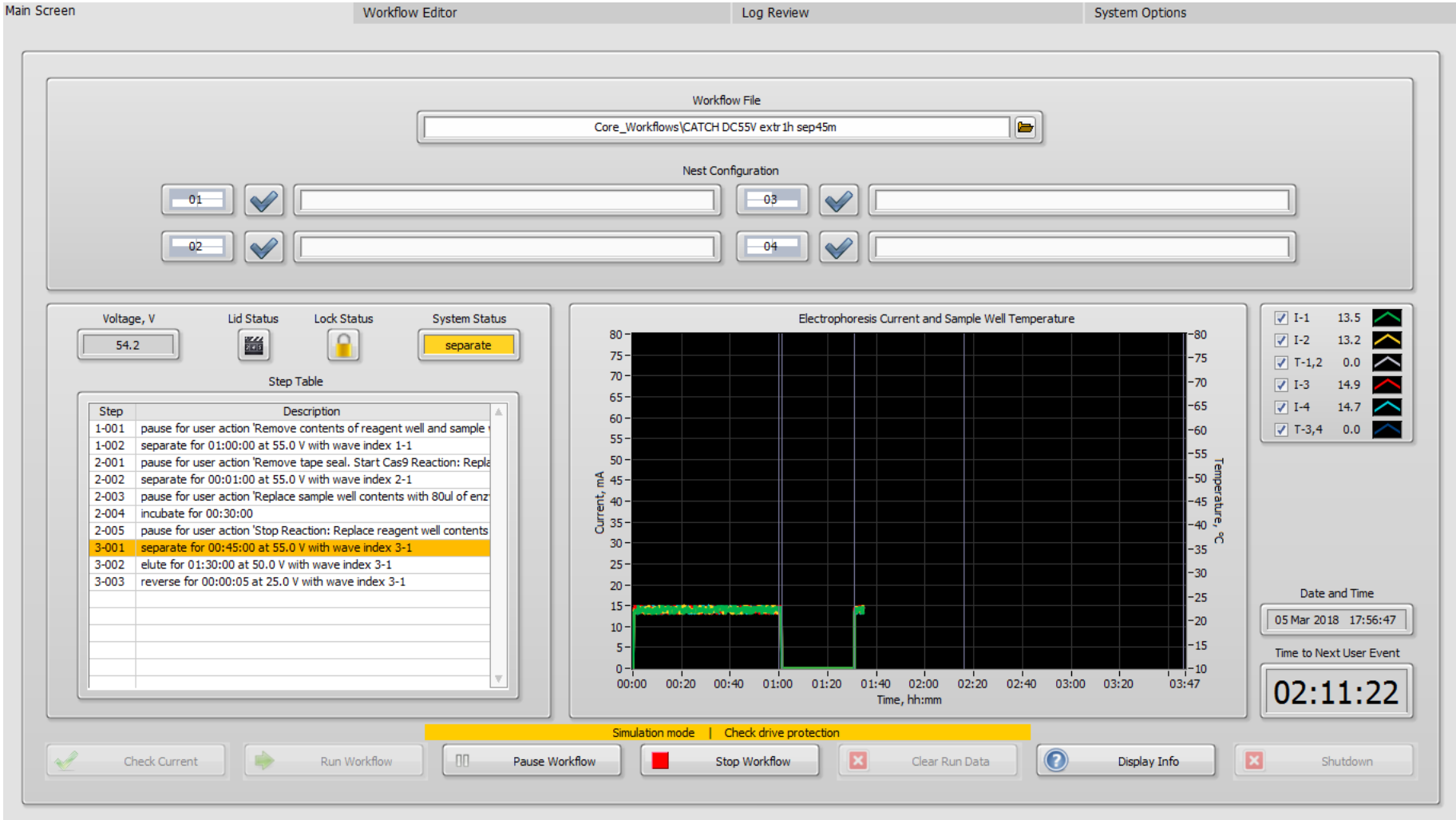
Screen after completion of Cas9 digestion.

Prompt will appear: “Stop reaction…”.
Empty Reagent well, and refill it with Lysis Buffer.
Carefully tape reagent, sample, and elution ports as for Stage 1. Close lid.
Click “OK” to start Stage 3 - Collection.



Main Screen after initiation of Stage 3

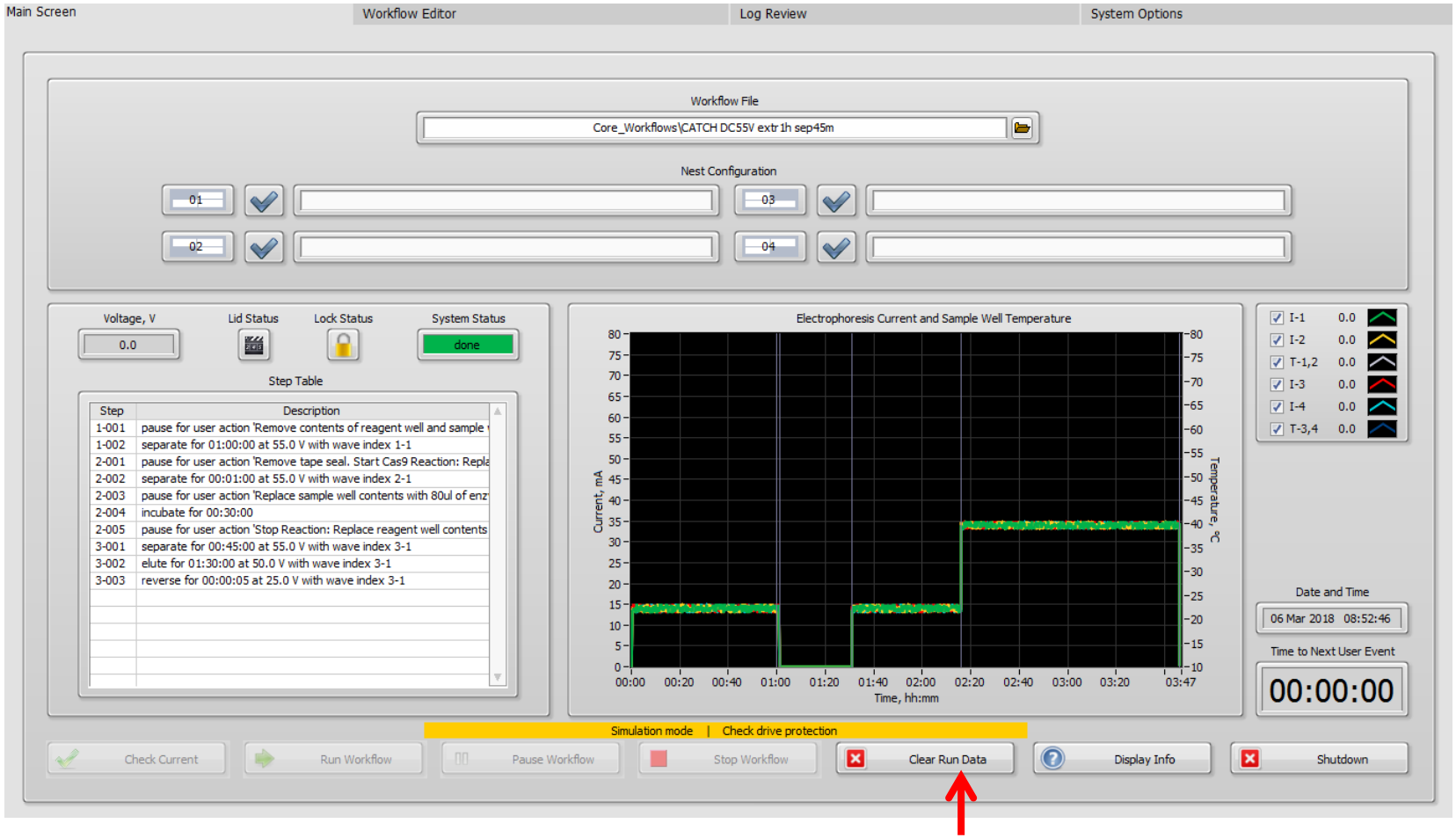
In this example, a very short Stage 3 protocol is shown. Other Stage 3's may be as long as 9.5 hrs.



HLS-CATCH run completed

This run was simulated in software, so current traces may not be so smooth in a real run. Also, the separation stage used here was very short, other separations may be significantly longer.

IMPORTANT: To program next run, you must “Clear Run Data” from previous run.



Completion of HLS-CATCH Workflow

If possible, remove the eluted DNA within an hour of the end of the run to avoid contamination of the eluted DNA with SDS. The SDS is tightly localized around (+) separation electrode at end of run, but will slowly diffuse throughout the cassette over a period of several hours.

Remove port sealing tapes by pulling the sealing tape tabs in a diagonal direction as discussed previously. (Removing the tape in this manner reduces the risk of introducing liquids from the Reagent or Sample well ports into the elution modules.)

If targeting DNA >100kb, use a wide-bore pipette tip to remove the contents of the elution modules.

IMPORTANT: When working with DNA >100kb, pipette as slowly as possible to avoid shearing the HMW DNA. Use of an electronic pipettor at low speed settings may be helpful.

There should be 70-80 ul of liquid in each module.

Analysis of HLS-CATCH products

To identify the position of CATCH targets in the elution products, we recommend the following procedures:

1) Perform a Qubit HS assay to determine total DNA content. (Use 2-5 ul aliquots of elution product.)
2) Perform a qPCR assay on all 6 elution products for CATCH target and non-target (control) genomic sequences to obtain target copy numbers for each EM.

The Qubit assay will give a semi-quantitative evaluation of the quality of the CATCH procedure. When using the 3hr Stage 1 extraction, there is very little total DNA in elution modules 1-5, usually ~2 ng per 80ul, while there will be more in EM #6, sometimes 20-30 ng per 80ul. When using the 1 hr Stage 1 extraction, there will be at least 10 ng of DNA (range 10ng to 200ng) in all elution modules, with higher concentrations in EM #1-3 (up to 200ng).

Whenever possible we prefer to use the "custom" Taqman qPCR assays available from Thermo Life Technologies, but also use the SYBR green kits for regions outside of genes. When using the 1 hour Stage1 extraction we typically see single-plex CATCH target enrichment factors of 15-50x relative to a single copy reference gene like the human RNaseP RNA gene. When using the 3 hr Stage 1 extraction with an input of 300,000-400,000 diploid mammalian cells, we have gotten enrichment factors as high as 700-fold.

Examples of CATCH target analyses: qPCR and Qubit HS data

200kb and 400kb targets from mouse Brca1 locus

375,000 WBC input load