sage science

SageHLS Cassette Kit Workflow Guide

HLS-CATCH
PN# HIT-0004 or HIT-0012

Reagents Supplied by Sage Science			Storage Temp.
4 / 12 ea.	Agarose gel cassettes		RT
20 / 60 ea.	Adhesive Tape Strips		N/A
1 ea.	HLS Lysis Reagent 3% SDS, 10 / 30 ml	A	RT
1 ea.	HLS Enzyme Buffer, 15 / 40 ml	C	4°C
1 ea.	Running Buffer, 40 / 115 ml	E	RT
1 ea.	4X Enzyme Buffer (for Cas9-Guide RNA Mix), 1 ml	F	-20°C
1 ea.	HLS Lysis Reagent 3% Sarkosyl, 10 / 30 ml	G	RT
1 ea.	HLS Lysis Reagent 1% SDS, 10 / 30 ml	H	RT

Materials Supplied or Prepared by User	Supplier	Cat#
S.pyogenes Cas9 enzyme, wild type:	New England Biolabs (NEB)	**M0386T** (400 pmol) **M0386M** (2,000 pmol)
Guide RNAs (crRNAs and tracrRNA)	Integrated DNA Technologies(IDT)	custom
Qubit™ Fluorometer/HS DNA Assay kit	Thermo Fisher Scientific	**Q32851** (100 assays)
TaqMan™ RNase P Detection Reagents Kit	Thermo Fisher Scientific	**4316831** (100 reactions)
TaqMan™ Target Detection Reagents	Thermo Fisher Scientific	custom

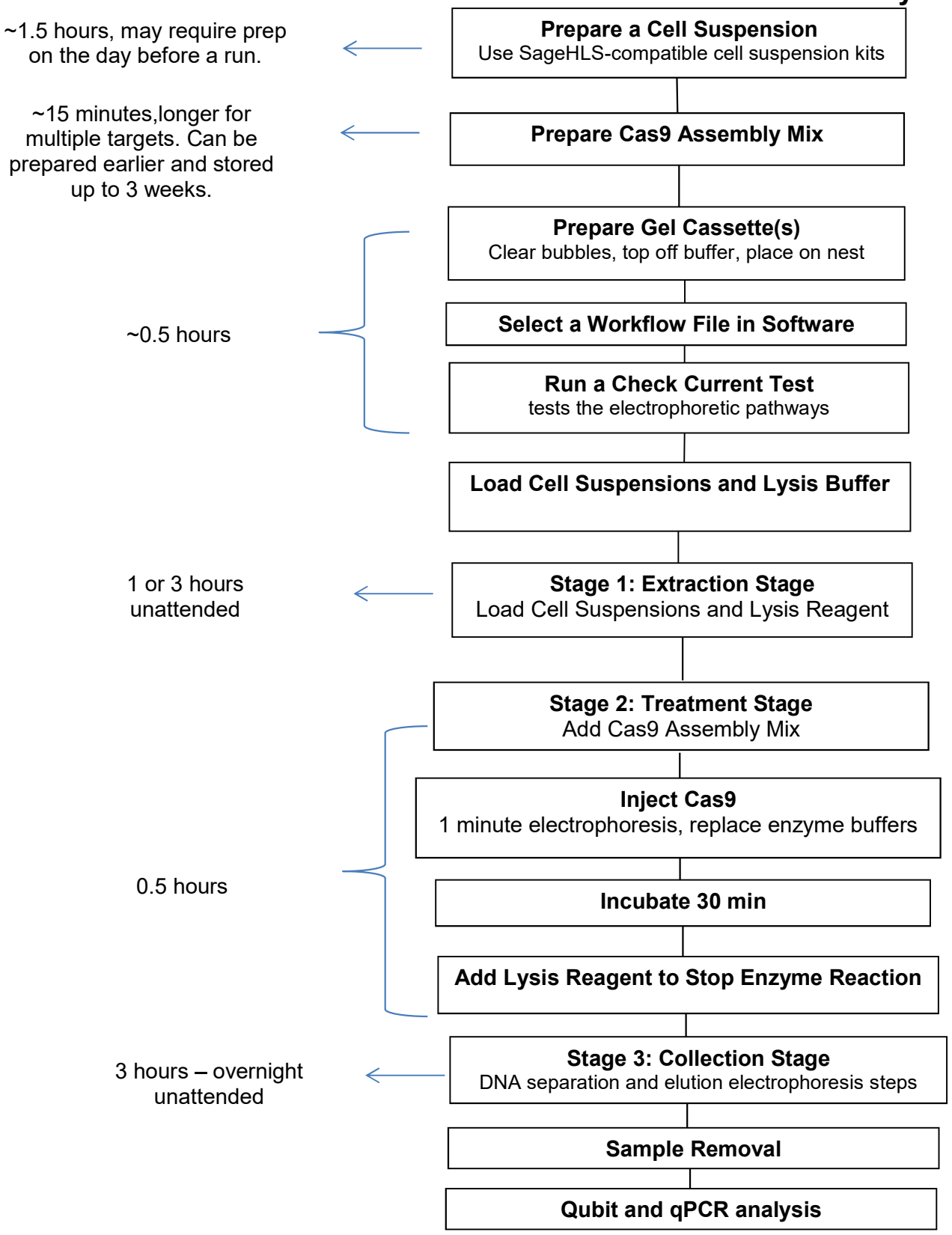
Important!

- Prior to day of extraction, schedule availability of cells. Check that cell preparation reagents are ready. Prepare cells using Sage HLS cell prep kit instructions.
- Recommended cell load per lane for HLS cassettes contains **2.5 µg of genomic DNA per lane**, (for example ~375,000 human diploid cells) in a maximum loading volume of **70 µl**. Higher cell loads will result in lower recovery of genomic CATCH targets.
- Assembly of Cas9 require about 30 minutes of effort and should be prepared before starting a SageHLS run. It may also be prepared up to four days in advance and stored at 4°C. Users should take Cas9 reagent preparation time into consideration when planning HLS procedures.
- In addition to the design of Cas9 guide RNAs, users should design and order primer sets and reagents for qPCR quantitation of enriched targets.

HIT kit Workflow Guide
Revision Change Log

Last Rev	New Rev	Date	Page#	Notes
C1	D	12/12/17	13-2	Added Revision Change Log
D	E	2/8/18	13-3	Extraction stage in workflow chart changed from 3hr to 1-3 hr
D	E	2/8/18	13-12	Added an optional step at the end of DNA extraction for removal of SDS in the buffer chamber around the anode
D	E	2/8/18	13-15 13-16	Added step to replace Enzyme buffer in the Reagent well in addition to the well after Cas9 injection
E	F	6/18/18	13-1	Change DNA equivalency load recommendation from 10ug to 2.5 ug
E	F	6/18/18	13-3,4,5	Revised Cas9 guideRNA assembly procedure
E	F	6/18/18	13-9,10	New workflow file naming conventions and descriptions
E	F	6/18/18	13-18	New step to (remove running buffer) to reduce SDS in eluants
E	F	6/18/18	13-23	New step to replenish running buffer after the treatment stage
E	F	6/18/18	13-9,27	Modified extraction times – 1 hr for 10X platforms, 3 hr for PacBio and Oxford platforms
E	F	6/18/18	13-27,28	Added description of Qubit and qPCR analyses
F	G	8/17/18	13-6	Replaced buffer chamber image to show even fill line.
G	H	10/24/18	13-4 13-5	Removed Cas9 gRNA assembly -20C storage recommendations. Not verified at that temperature.

HLS-CATCH Workflow Summary



A. Prepare cells using Cell Suspension Guide

Prepare cells using SageHLS 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.

B. Prepare the Cas9 Enzyme Assembly (can be done in advance)

The Cas9 assembly can be prepared ahead of time and stored up to 4 days at 4 °C without loss of activity. The mix can be stored on ice (thawed first, if necessary) prior to use.

Summary

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

1. Anneal the two-part IDT ALT-R™ guide RNAs (crRNA and tracrRNA)

a. 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.

b. Use the table below to prepare the Guide RNA Annealing Mix to a volume of 22 ul for the preparation of one sample. Users should scale accordingly if the same guides will be used for multiple sample treatments.

To a 200 µl PCR tube, add:

order of addition	reagent	vol. µl	stock [ ] µM	Final [ ] Annealed gRNA Mix µM
1	**IDT Duplexing Buffer**	15.4		
2	**Pooled crRNAs***	4	100	18.2
mix and spin				
4	**tracrRNA**	2.6	100	11.8
mix and spin				
	Total	**22**		

*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 is to saturate the tracrRNA with a 1.5-fold molar excess of total crRNA**.)

d. Remove the mix from heat and allow to cool on the bench-top for 3-5 minutes.

e. Centrifuge for 30-60 seconds to collect any condensation.

f. Stored up to 4 days at 4 °C.

2. Assemble Annealed Guide RNAs and Cas9 mixture

a. Make sure the frozen reagents have been thawed: 4X Enzyme Buffer (**F**), NEB Cas9 nuclease

b. Using the following order of addition, assemble the gRNA-Cas9 reaction mixture:

To a 200 µl PCR tube, add:

order of addition	reagent	vol. µl	stock [ ] µM	final [ ] in Enzyme Mix µM
1	4X Enzyme Buffer (**F**)	10	4	1
2	Annealed Guide RNA Mix	22	11.8 (tracrRNA)	6.5 (tracrRNA)
mix and spin				
3	NEB Cas9 nuclease, wt	8	20	4
	Total*	**40**		

*40 ul will be diluted to 80 ul before loading in a single sample well. Users should scale accordingly.

c. Mix by pipetting up and down.

d. Incubate at 37°C for 10 minutes in a thermal cycler with a heated lid to assemble enzyme and gRNAs.

e. Proceed to next step (Dilution with HLS Enzyme Buffer) or place on ice until ready.

3. Dilute to loading concentration with HLS Enzyme Buffer

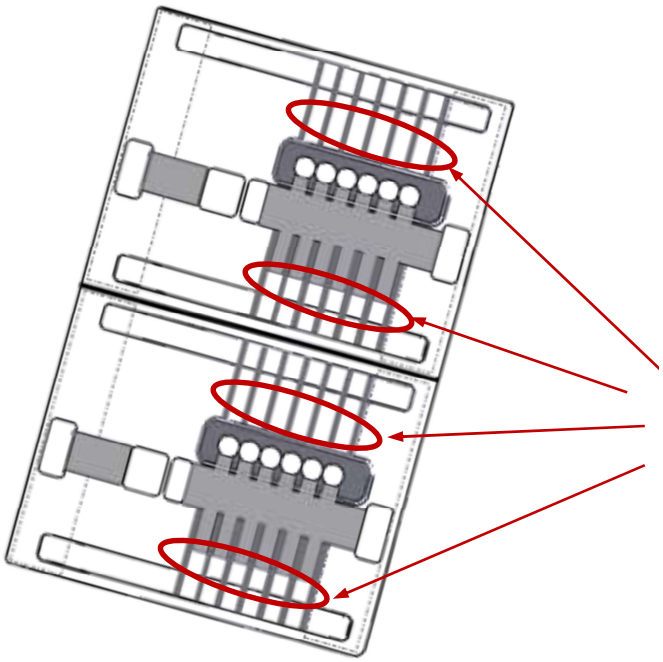
Dilute the assembled Annealed Guides/Cas9 mixture from Step 2 (40ul), with 40 ul of HLS Enzyme Buffer (**C**). Mix thoroughly by gentle pipetting of the entire mixture (80 ul). (Final conc: 2 uM Cas9 assembled with 3.25 uM pool of annealed two-part gRNAs)

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.

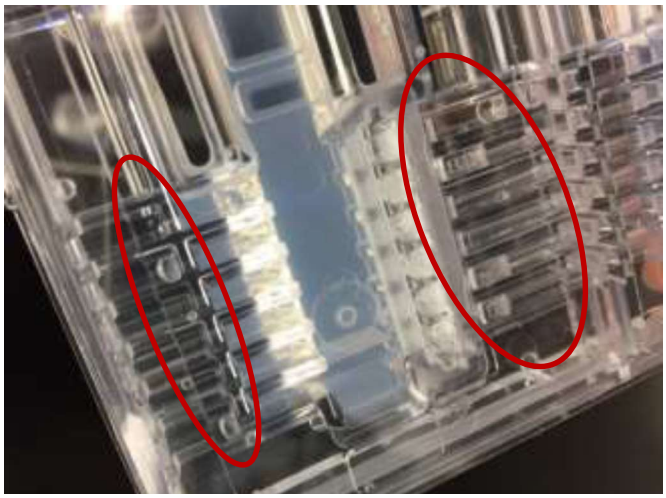
C. Prepare the Gel Cassette(s)

1. Remove the gel cassette from the foil bag.

2. **Before removing tape! –** Hold the cassette with top surface almost vertical and the elution modules above the gel channel. Tap the cassette to dislodge any bubbles that are trapped behind the elution wells, or in the elution channels. Repeat if necessary. Allow the bubbles to collect in the electrode channel directly above.

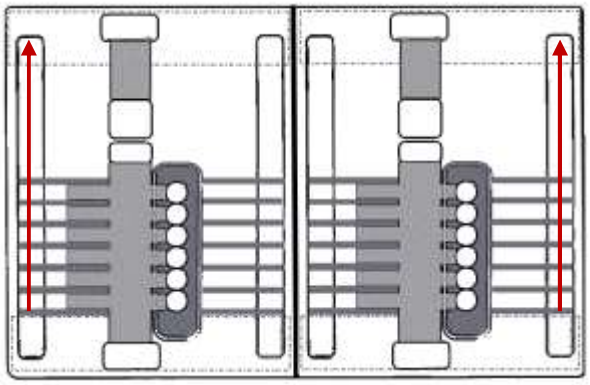


Tap to dislodge bubble from these areas into the troughs directly above.

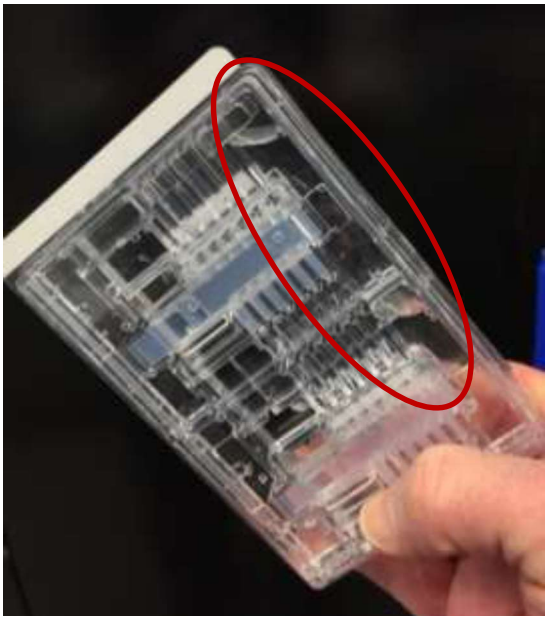


Bubbles in the elution paths can interfere with collection

3. Slowly rotate the cassette to allow the bubbles to collect in the upper buffer area. Gently tap if necessary.



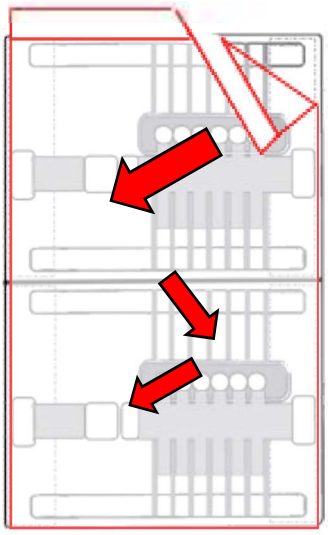
Move any bubbles to the upper buffer area.



Aggregate and collect air bubbles in the upper buffer chambers

4. With cassette held at a slight angle to keep bubbles located in the upper buffer chamber, gently place the cassette onto the nest. The upper buffer chamber should be on the left side of the nest.

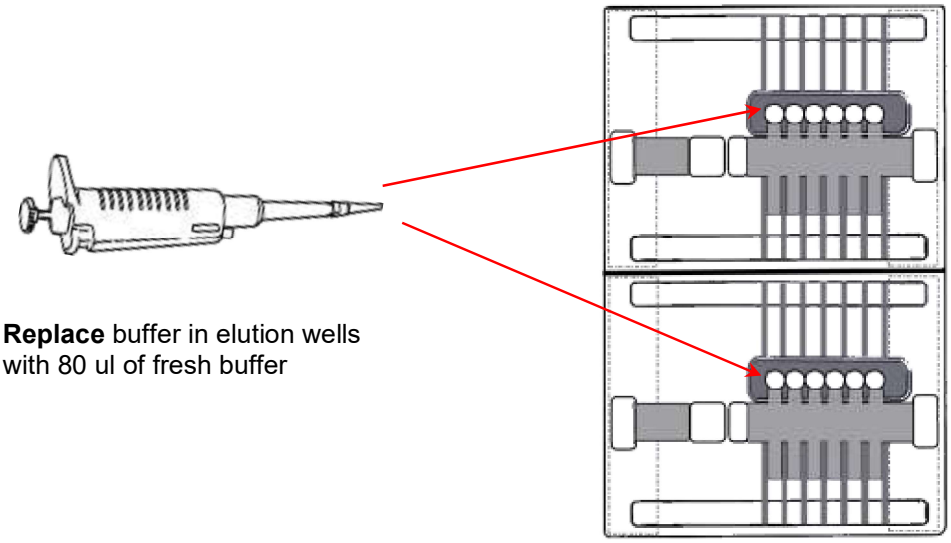
5. While holding the cassette firmly in place on the nest, grab the tape tab, and pull the tape off at an angle, slowly and firmly. Alternate the pulling angle if the tape resists peeling.



Peel back tape at an angle. Alternate angles if the tape resists peeling.

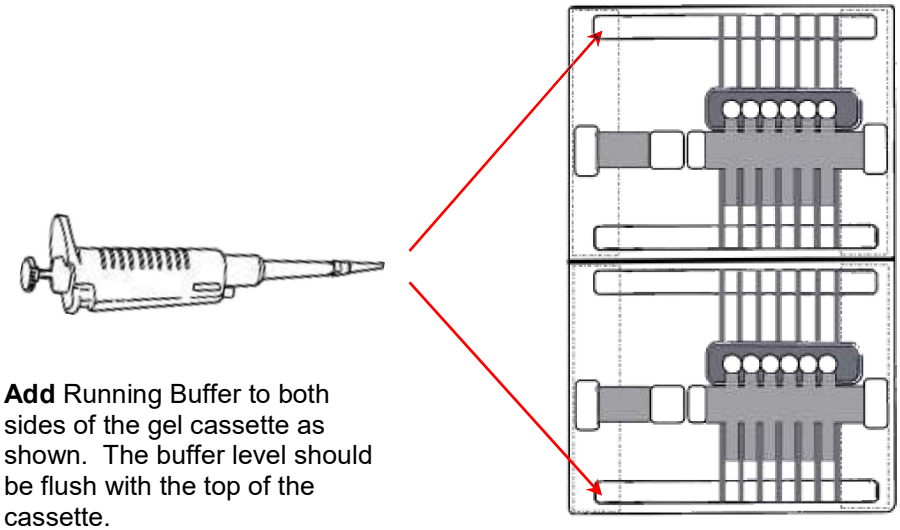
6. Remove all buffer from all elution wells (set a pipette to 100 µl to completely empty the wells). Keep pipette tips vertical in the well to avoid damage to the membranes.

7. Taking care not to introduce additional bubbles into the elution modules, add 80 µl of Running Buffer (**E**) to all elution wells.



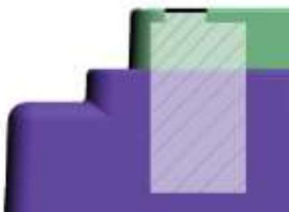
Replace buffer in elution wells with 80 ul of fresh buffer

8. Add **Running Buffer** to the upper buffer chamber on each side of the gel cassette until the level is flush with the top of the cassette. An adequate buffer level is important for achieving best results from the SageHLS.



Add Running Buffer to both sides of the gel cassette as shown. The buffer level should be flush with the top of the cassette.

Fill buffer until it is flush with the top of the cassette



Important! Fill until the buffer level visually reaches the bottom side of the cassette cover

D. Load the Workflow File

Workflow File Guidelines:

There are two types of workflow files that are based on level of method validation at Sage Science:

Core Workflows – Tested extensively on biological samples
Non-core Workflows – Tested with model DNA sample input only

File Naming Conventions
For convenience, Sage-provided workflow file names follow the following convention (if practical):

[Application type]_[DNA Size Target]_[DNA extraction time]_[DNA separation time].shflow

Example: **CATCH 100-300kb extr 1h sep 3h.shlfow**

Selecting DNA Target Sizes
Core workflows have been tested for targets of 100, 200, and 400kb ("100-300kb" files) and smaller targets up to 30kb ("55V" files). Although all elution wells should be interrogated for enriched product (see pages 13-27 and 13-28), workflows are designed to collect targets in well 3 and 4. Longer separation times will collect larger targets in the same wells:

≥ 100kb: use "100-300kb"
≤ 30kb: use "55V"

Selecting Extraction Times
Variable amounts of non-specifically cleaved DNA (50kb to 200kb) can be liberated from non-viable cells during the extraction step. With a one hour extraction, some of these fragments will co-elute with CATCH targets. These fragments benefit 10X Genomics linked-read library construction by providing a reference:

10X Genomics linked reads: use 1 hr extraction
Pac-Bio or Oxford nanopore: use 3 hr extraction

Selecting Separation Times
For a given target range, a longer separation time will collect larger DNA targets in the same elution wells. The following targets have been extensively tested using following core workflows:

100kb: 3 hour separation (CATCH 100-300kb extr 3h sep 3h.shlfow)
200kb: 3 hour separation (CATCH 100-300kb extr 3h sep 3h.shlfow)
400kb: 3 hour separation (CATCH 100-300kb extr 3h sep 4h.shlfow)
16kb: 45 min separation (CATCH DC55V extr 3h sep 45m.shlfow)
30kb: 75 min separation (CATCH DC55V extr 3h sep 75m.shlfow)

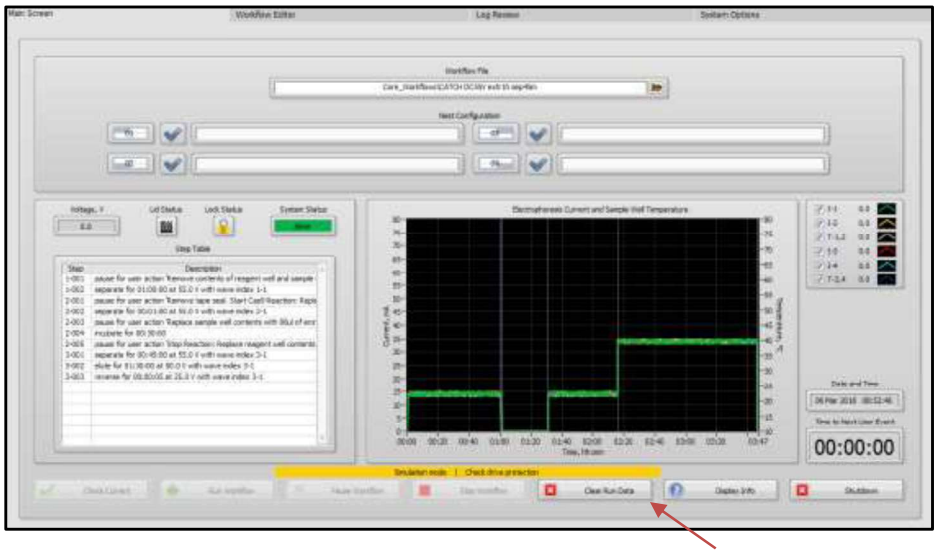
Workflow File Summary

Workflow Files	
Core Workflows	**~ Run Time (hr)**
CATCH 100-300kb extr 1h sep 3h.shlfow	6
CATCH 100-300kb extr 1h sep 4h.shlfow	7
CATCH 100-300kb extr 3h sep 3h.shlfow	8
CATCH 100-300kb extr 3h sep 4h.shlfow	9
CATCH DC55V extr 1h sep 45m.shlfow	4
CATCH DC55V extr 1h sep 75m.shlfow	4.5
CATCH DC55V extr 3h sep 45m.shlfow	6
CATCH DC55V extr 3h sep 75m.shlfow	6.5
Non-Core Workflows	**~ Run Time (hr)**
CATCH 100-400kb extr 1h sep 3h.shlfow	6
CATCH 100-400kb extr 1h sep 4h.shlfow	7
CATCH 100-400kb extr 3h sep 3h.shlfow	8
CATCH 100-400kb extr 3h sep 4h.shlfow	9
CATCH 145kb extr 1h.shlfow	11
CATCH 145kb extr 3h.shlfow	13
CATCH 200kb extr 1h.shlfow	11
CATCH 200kb extr 3h.shlfow	13
CATCH 400kb extr 1h.shlfow	11
CATCH 400kb extr 3h.shlfow	13

About non-core workflows

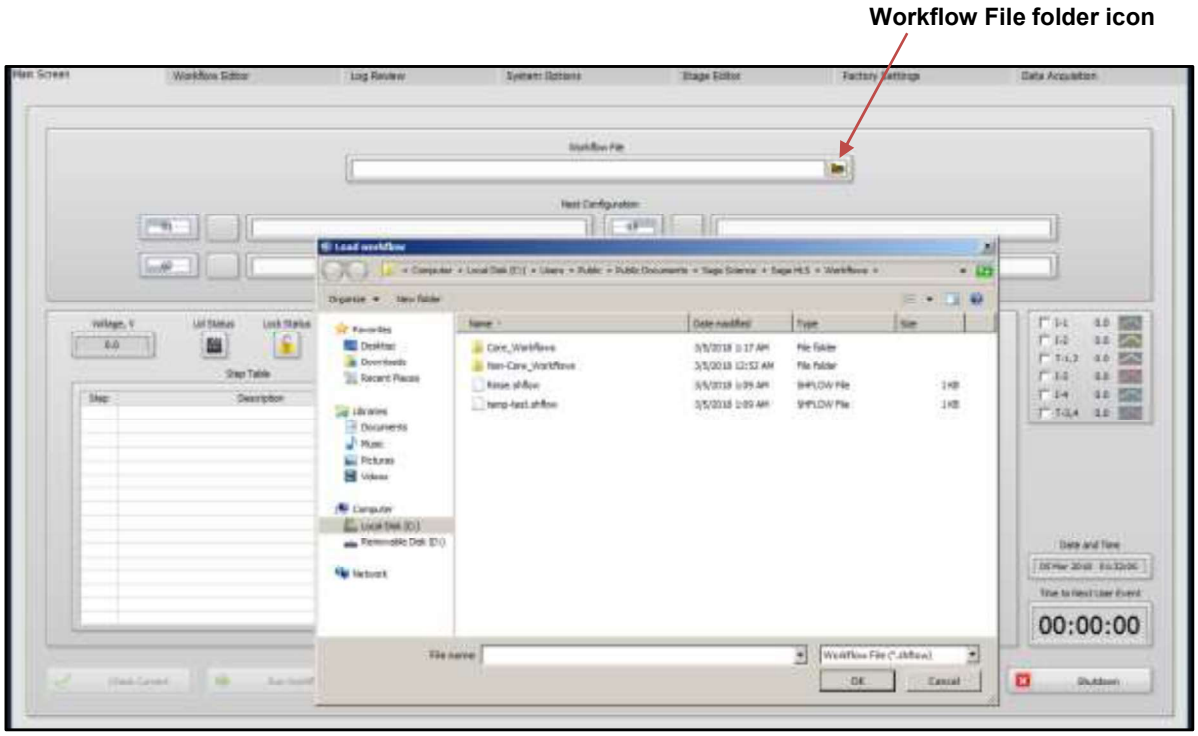
Non-core workflows have been designed for special purposes. For instance, the CATCH 100-400k workflows are designed to have a broader collection range than the core 100-300kb workflows, whereas the “CATCH <xxx>kb” workflows are designed to have expanded resolution in a narrow window centered on <xxx>kb.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

1. Go to the Main screen of the SageHLS software. If there is data from a previous run, the "Clear Data" button must be pressed to clear all fields.

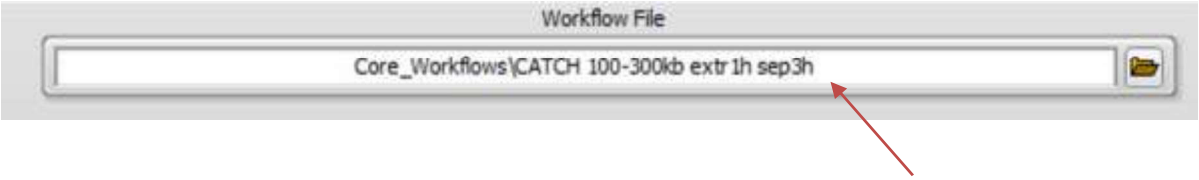


2. Select the Workflow File folder icon.

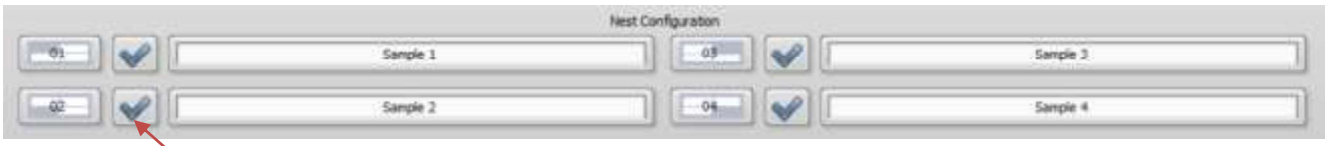
   A workflow file directory window will open, select the folder containing either core or non-core workflows.



3. Select the Workflow File from the folder. The file name will appear in the Workflow File field.



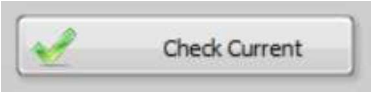
4. Choose the lanes to be used by clicking the boxes next to the lane numbers and enter sample IDs into the adjacent fields (sample IDs are optional or can be entered later).



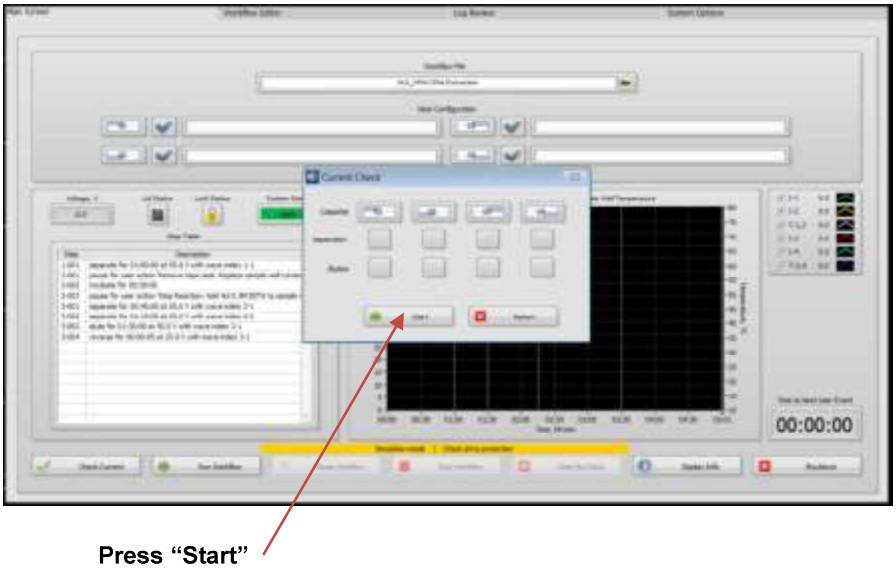
Check marks indicate which lanes are active

E. Run the Check Current Test

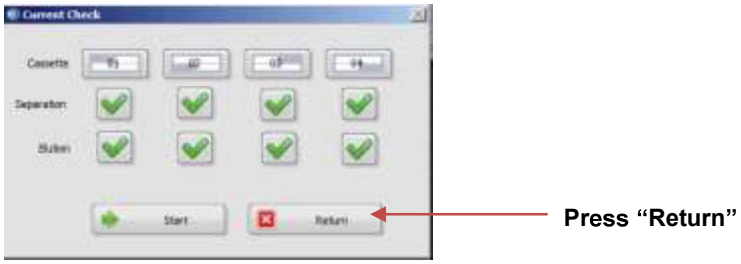
1. Press the “Check Current” button.



2. A pop-up window will appear. Press “Start” to begin the Check Current routine.



3. The routine will first test the separation electrodes, then test the elution electrodes, and complete within a few minutes. After a successful test, all boxes will fill with green check marks. Press “Return” to continue

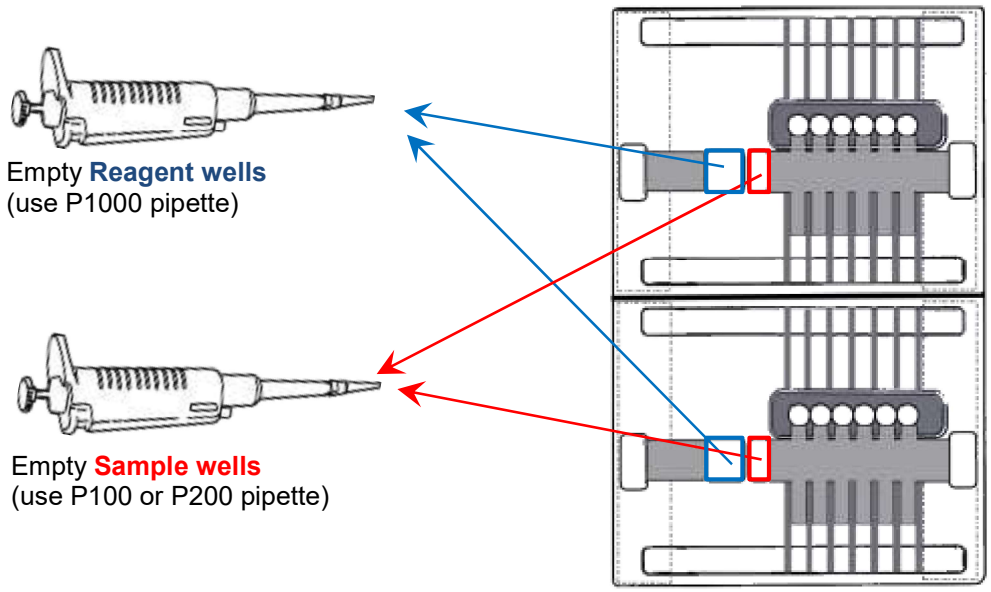


F. Stage 1: Extraction

1. Use the following Table as a guide to select the most appropriate Lysis Reagent.:

Lysis Reagent Name	Description
HLS Lysis Reagent 3% SDS (**A**)	Maximum DNA recovery, residual SDS (0.01-0.03%) in eluant
HLS Lysis Reagent 1% SDS (**H**)	Less DNA recovery (25-30%), less residual SDS (0.001 – 0.009%)
HLS Lysis Reagent 3% Sarkosyl (**G**)	Less DNA recovery (25-30%), for samples with potassium (alternative to SDS, which co-precipitates in the presence of potassium)

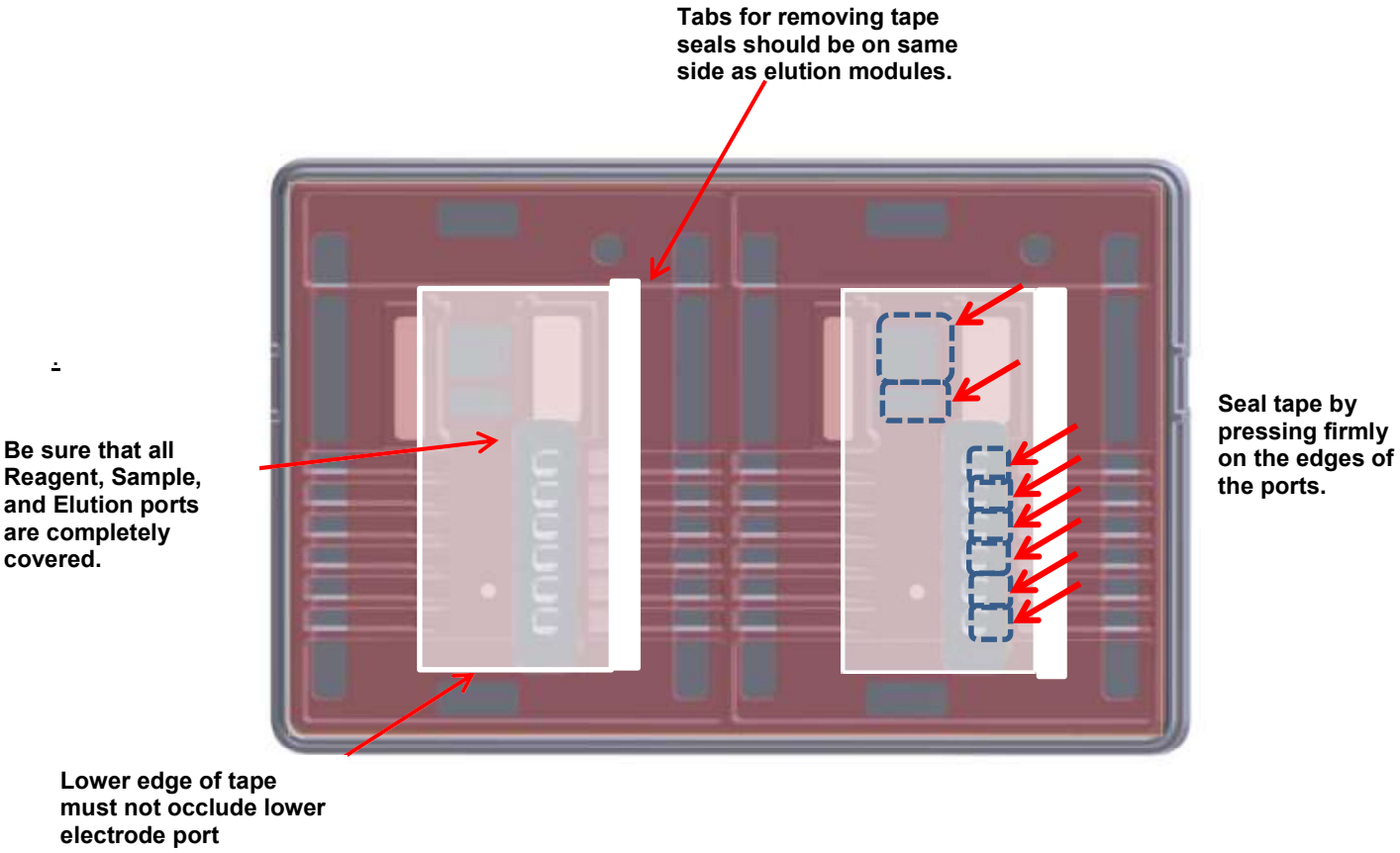
2. Empty all sample and reagent wells. Use caution not to pierce agarose at the bottom of the wells.



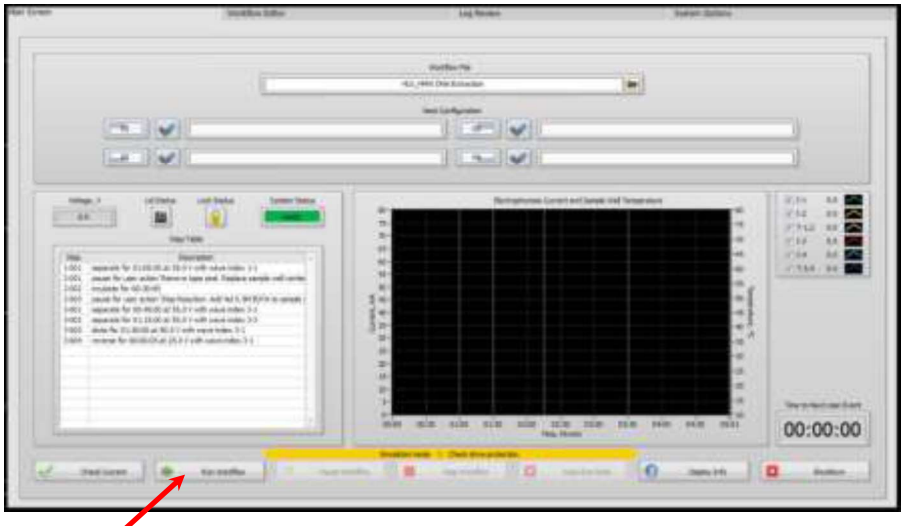
3. Load samples in all lanes. Use a **70ul sample loading volume**. (Sample wells will not be completely full.)

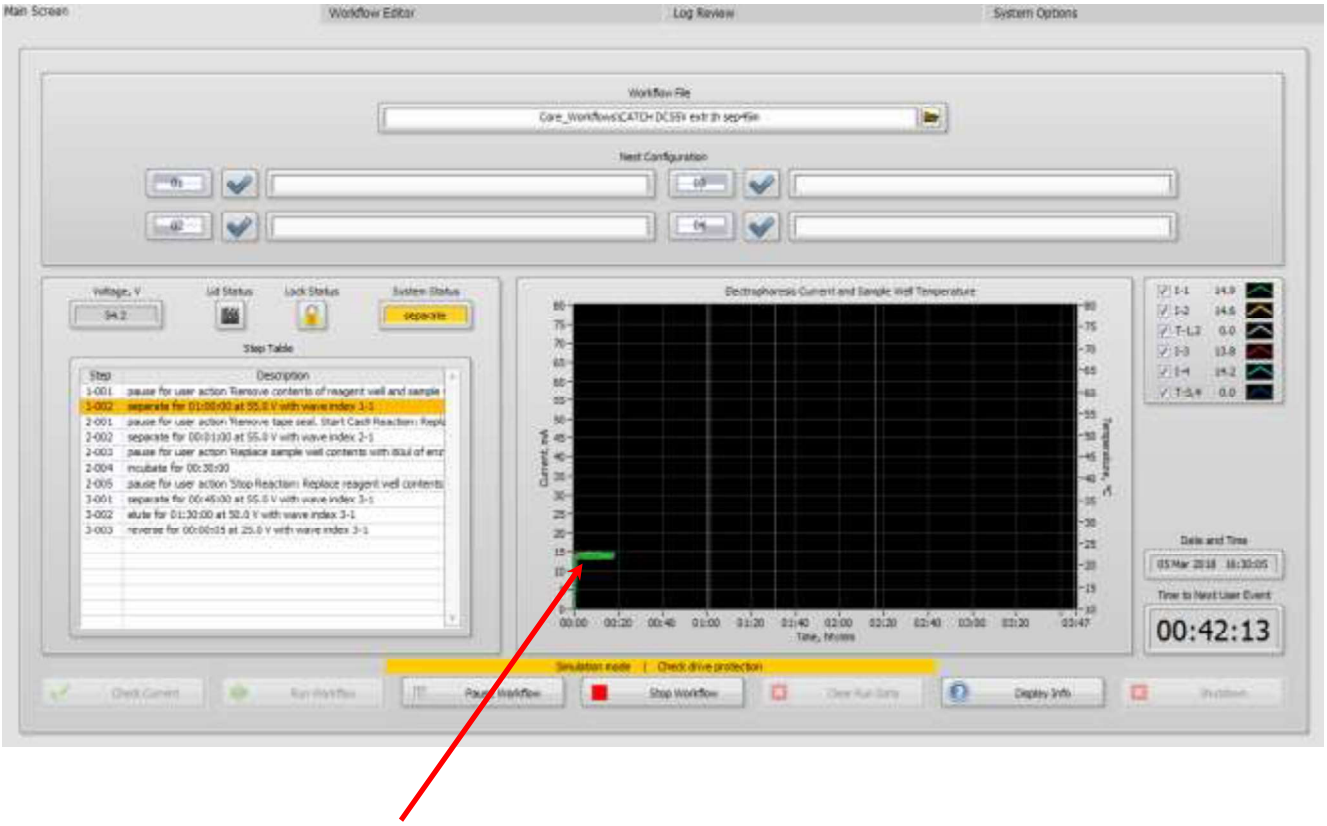
4. **Add 220 ul of HLS Lysis Buffer** (**A** or **G**) to the reagent Wells with. Do not overfill! Leave a concave meniscus to prevent contact with sealing tape in next step.

5. Seal reagent, sample, and elution ports with supplied tape without occluding the electrode ports. Press tape firmly around edges of the ports.



6. Close the lid and press “Run Workflow”. The extraction step will require the amount of time indicated in the workflow file name, typical 1 or 3 hours.

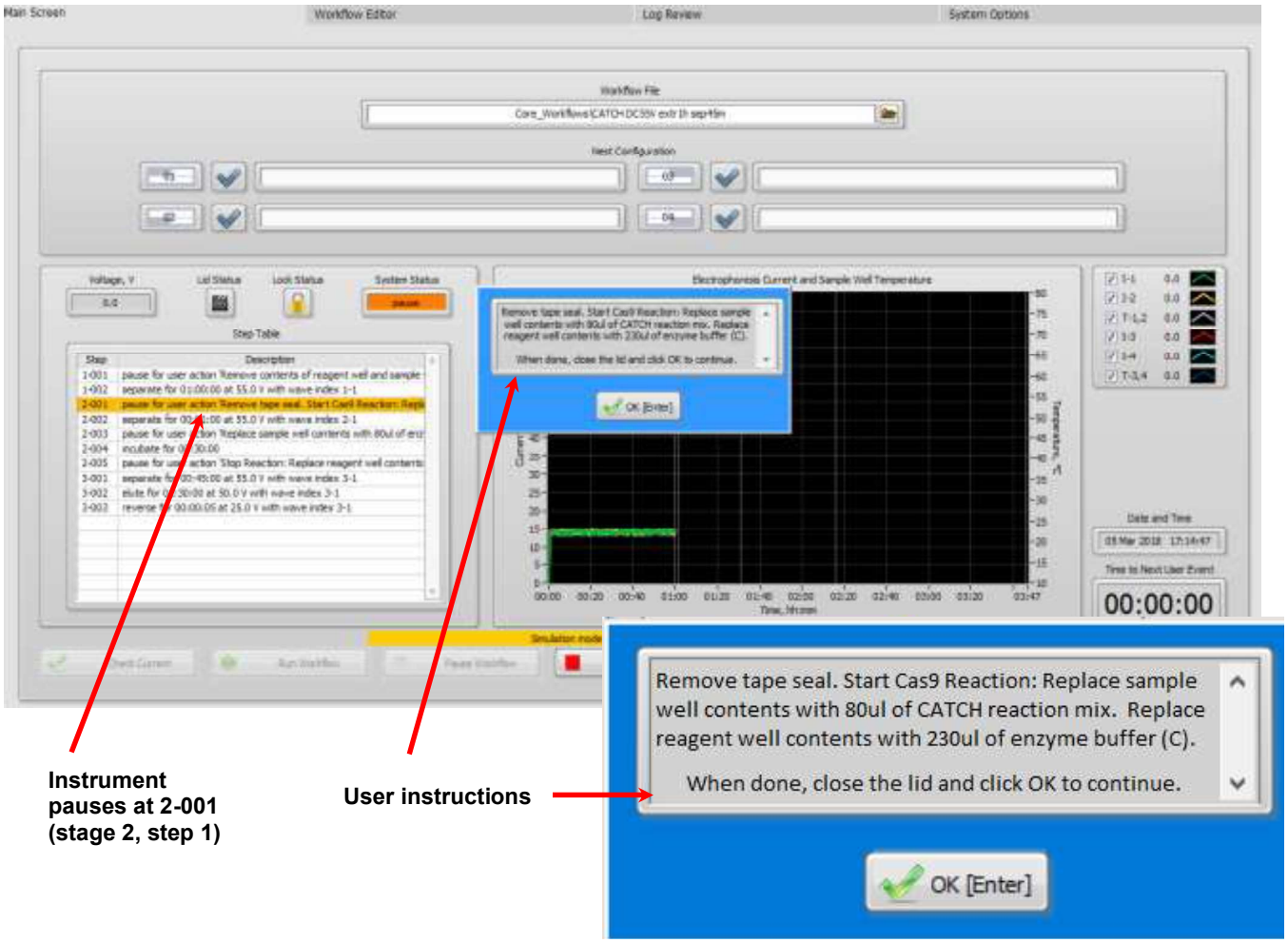




During the run, the Current display should be similar for all lanes, and in the range of 10-15 mA

G. Stage 2: Treatment

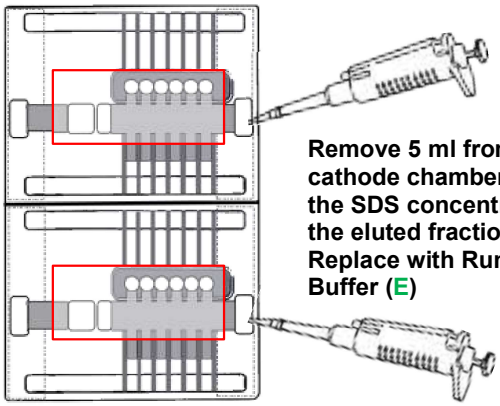
At the end of the Extraction Stage, the SageHLS will pause on the first step of the Treatment Stage and a pop-up window will appear with user instructions.



Important! The instrument will remain paused while the user action pop-up window is displayed. It is resumed when the "OK" button is pressed. If the instrument is inadvertently resumed, press the "Pause Workflow" button in the Command Menu to re-pause the instrument and continue with the manual user action.

1. Replace 5 ml of buffer from the anode chamber

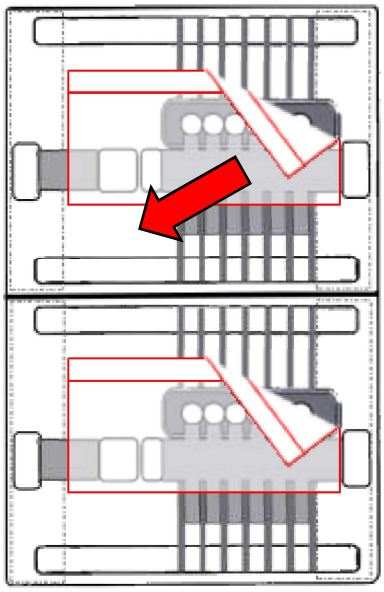
   Detergents (SDS, Sarkosyl) will aggregate near the electrode in the anode chambers of the gel cassette during extraction. By replacing buffer in this area immediately after the extraction stage, the detergent concentration will be reduced in the final eluted samples.



Remove 5 ml from the cathode chamber to reduce the SDS concentration in the eluted fractions. Replace with Running Buffer (E)

2. Open the lid and carefully remove the adhesive tape(s). To remove the tapes, grab the tab in right upper corner and peel diagonally with a slow smooth motion.

grab the tab in right upper corner and peel diagonally with a slow smooth motion



Important! Pulling in the tape in diagonal fashion prevents liquid transfer between adjacent elution ports and transfer between the sample/reagent ports and the elution ports.

3. Remove the contents from the Reagent wells and Sample wells on the cassette(s). The well volumes are 270 µl and 85 µl, respectively.

4. **Dilute 40 ul of the Cas9-Guide RNA Mix with 40 ul HLS Enzyme Buffer** (**C**) (80 ul total)

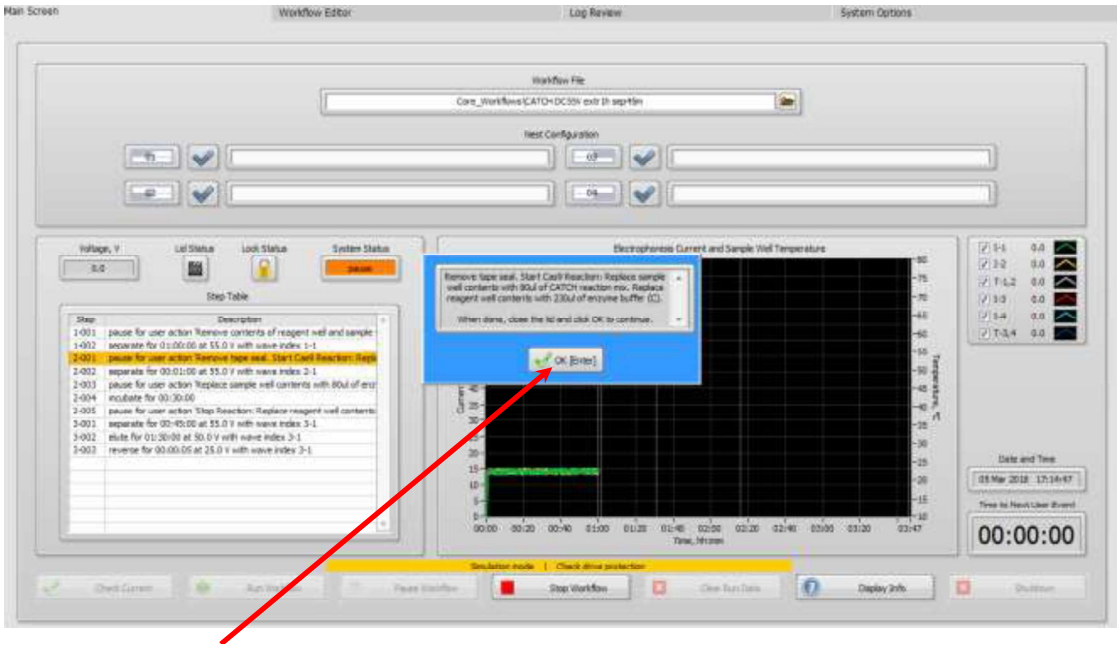
5. Mix thoroughly by pipetting up and down.

6. **Add 80 µl of the diluted Cas9-Guide RNA Mix** to the **Sample well(s)**.

7. **Add 230 µl HLS Enzyme Buffer (C)** to the **Reagent well**(s)

8. Close the lid (do not re-seal the wells with tape).

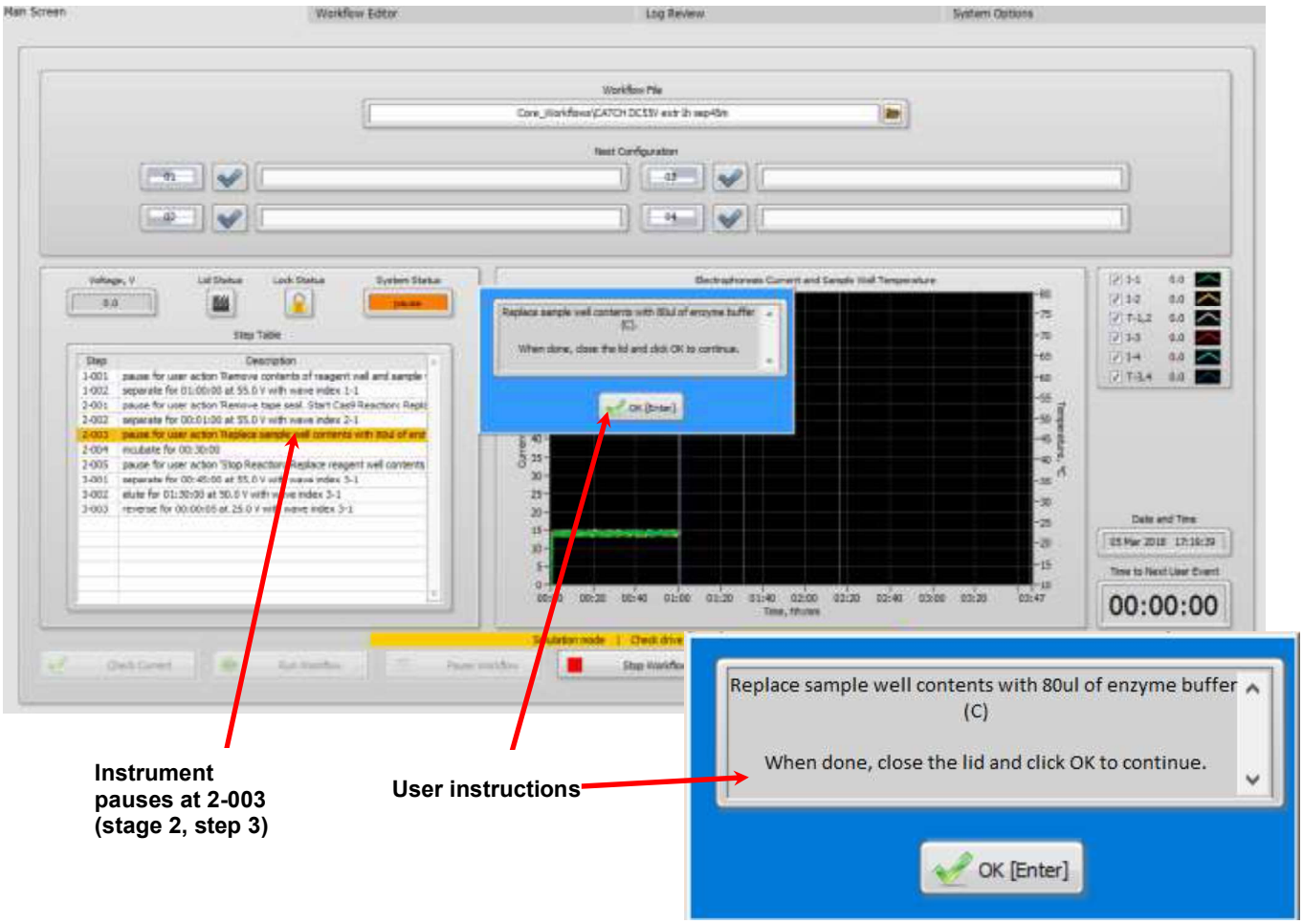
9. Press "OK" in the pop-up window to resume the workflow.



Press "OK" to resume

10. The instrument will perform a **1 minute electrophoresis step** to inject the Cas9 into the sample wall where the HMW DNA is immobilized.

11. The SageHLS will pause, and a pop-up window with user instructions will appear.



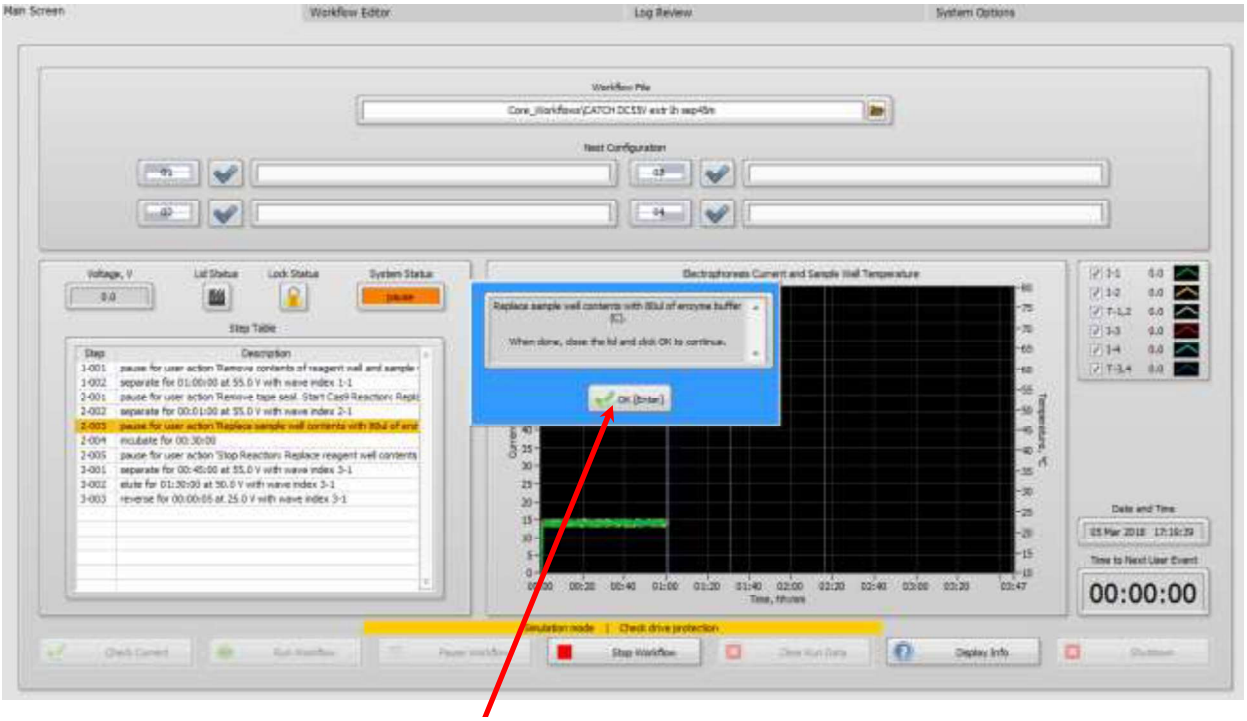
12. Open the lid.

13. Remove the contents from the **Sample well(s)** on the cassette(s).

14. Add **80 µl of the Enzyme Buffer** (**C**) to the **Sample well(s).**

15. Close the lid (do not re-seal the wells with tape).

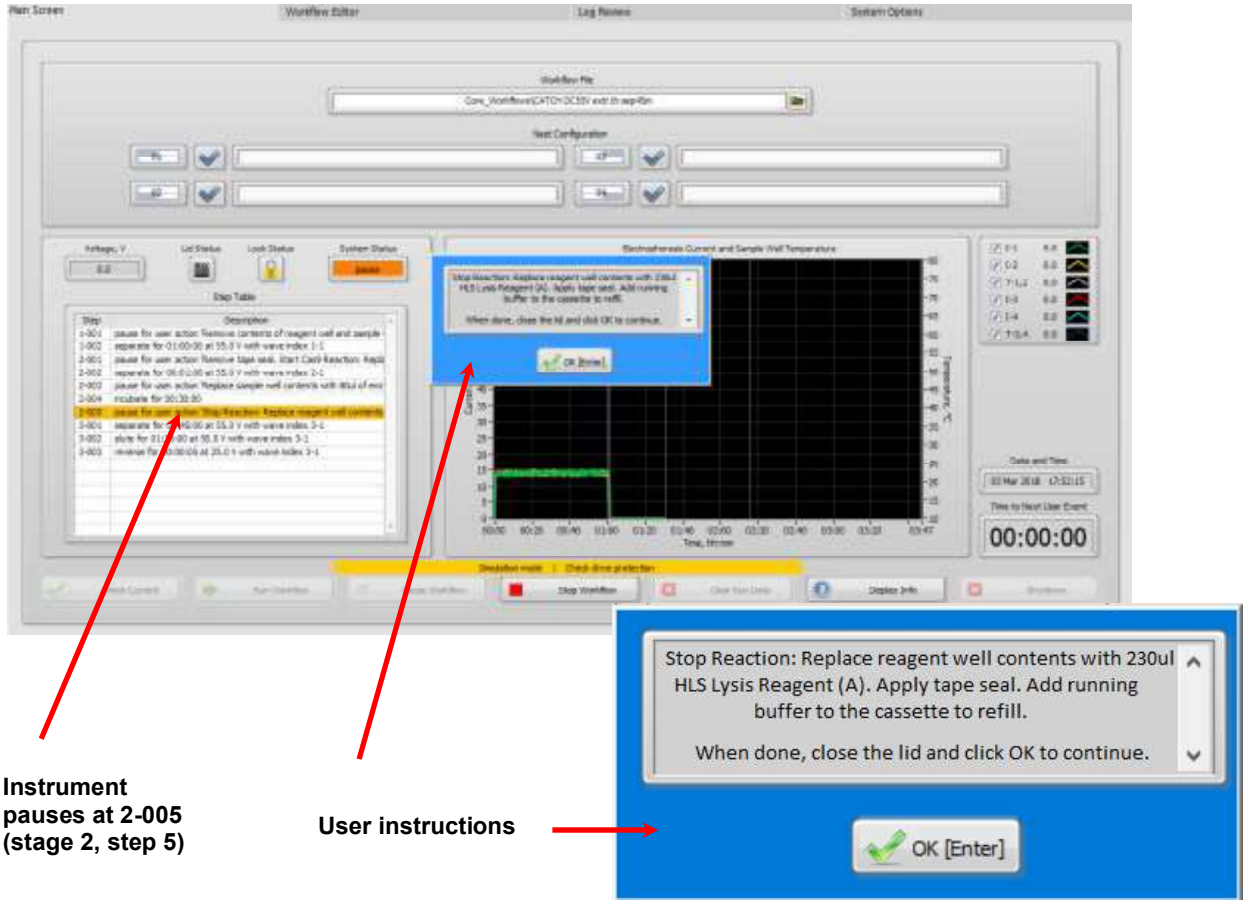
16. Press “OK” in the pop-up window to resume the workflow.



Press “OK” to resume

19. The enzyme treatment step will take **30 minutes**.

20. At the end of **30 minutes** the SageHLS will pause, and a pop-up window with user instructions will appear.

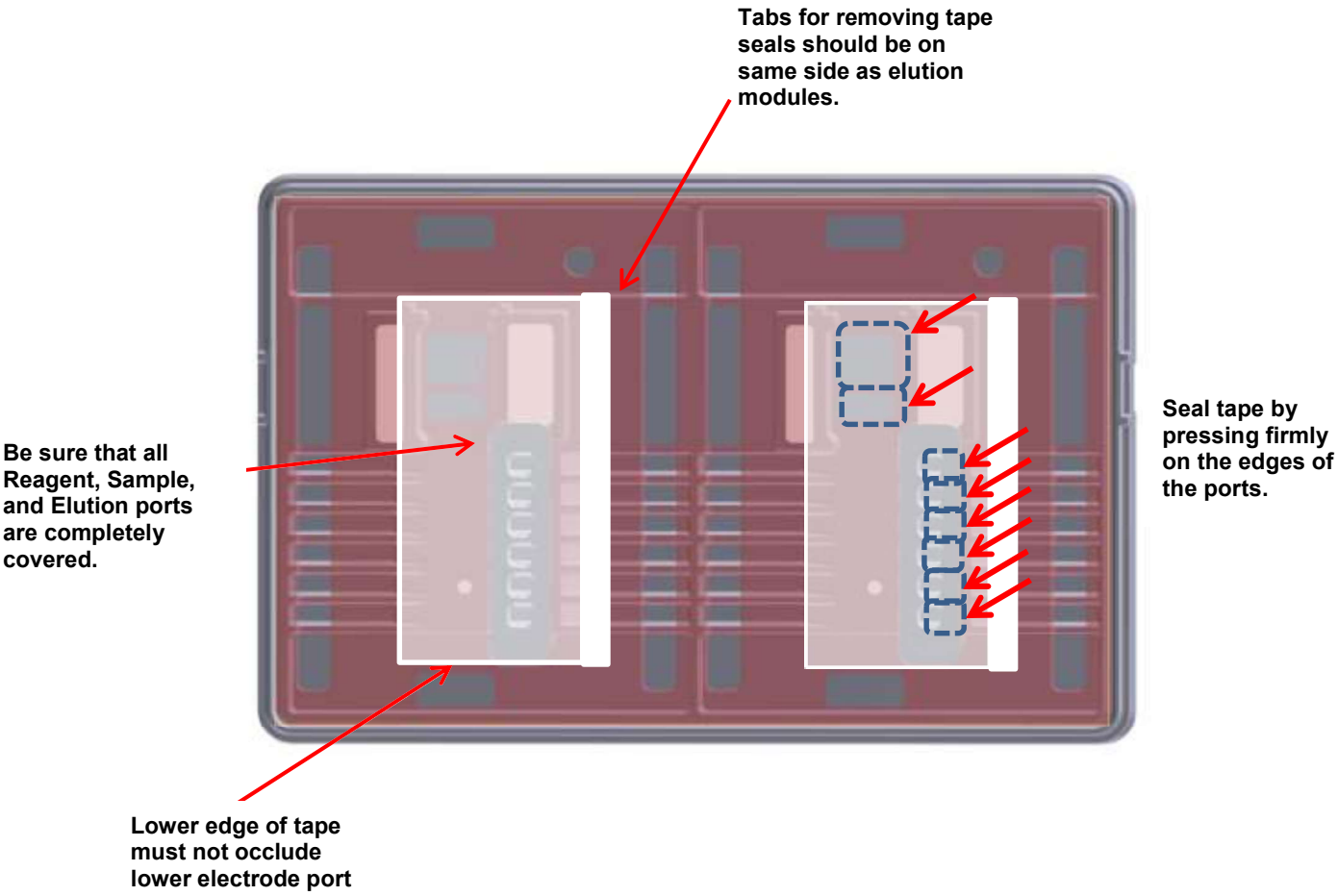


Important! The instrument will remain paused while the user action pop-up window is displayed. It is resumed when the "OK" button is pressed. If the instrument is inadvertently resumed, press the "Pause Workflow" button in the Command Menu to re-pause the instrument and continue with the manual user action.

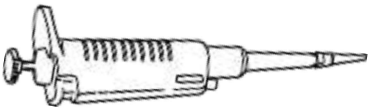
21. Open the lid and remove the contents of the Reagent well.

22. **Add 220 ul Lysis Reagent** (**A, H** or **G**) **to the Reagent well(s).** Do not overfill! Leave a concave meniscus to prevent contact with sealing tape in next step.

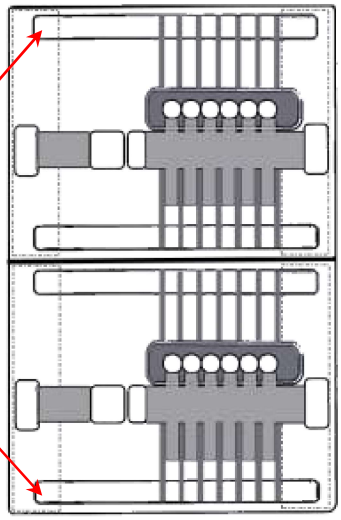
23. Close the lid and **re-seal the cassette wells with tape**.

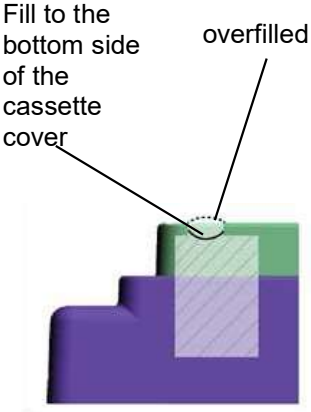


24. Add running buffer (**E**) to the cassette to replace evaporated buffer.

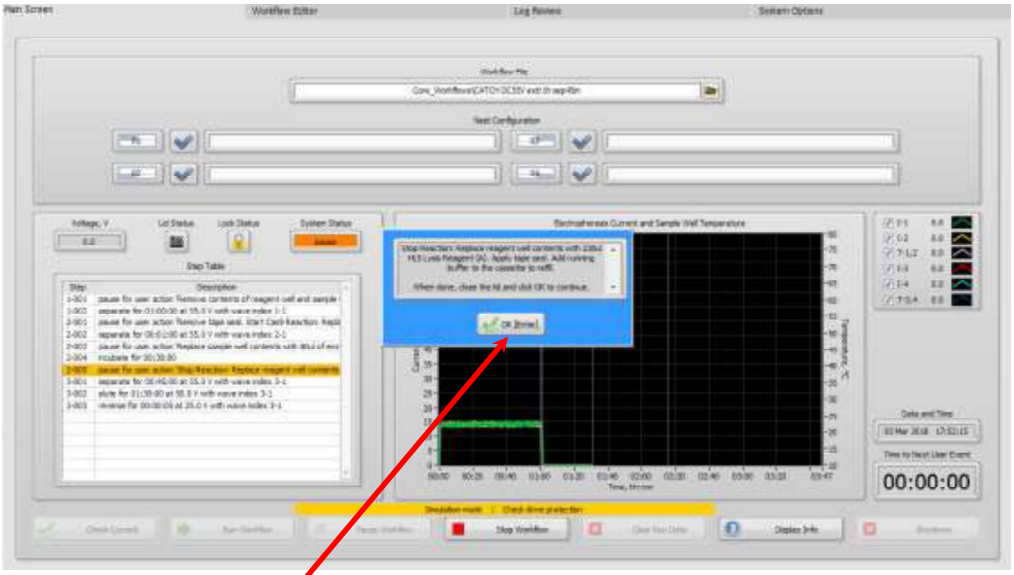


Add Running Buffer to both sides of the gel cassette as shown. The buffer level should be flush with the top of the cassette.



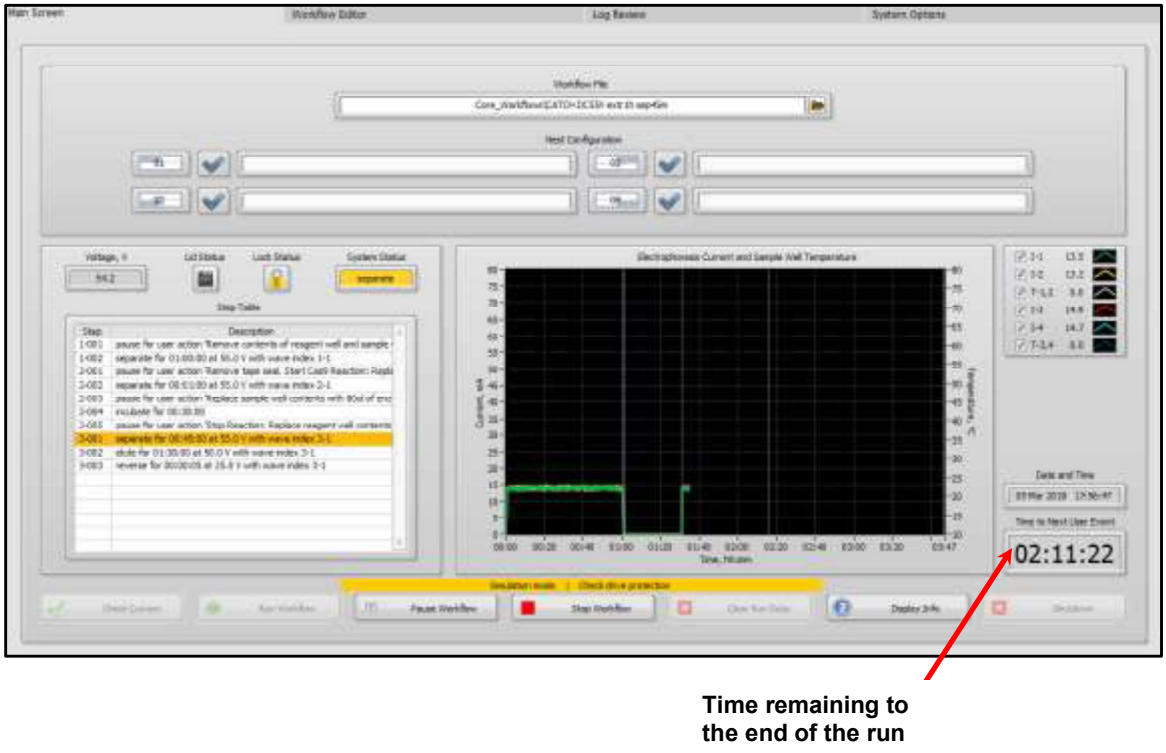


25. Close the lid and press “OK” in the pop-up window to resume the workflow.



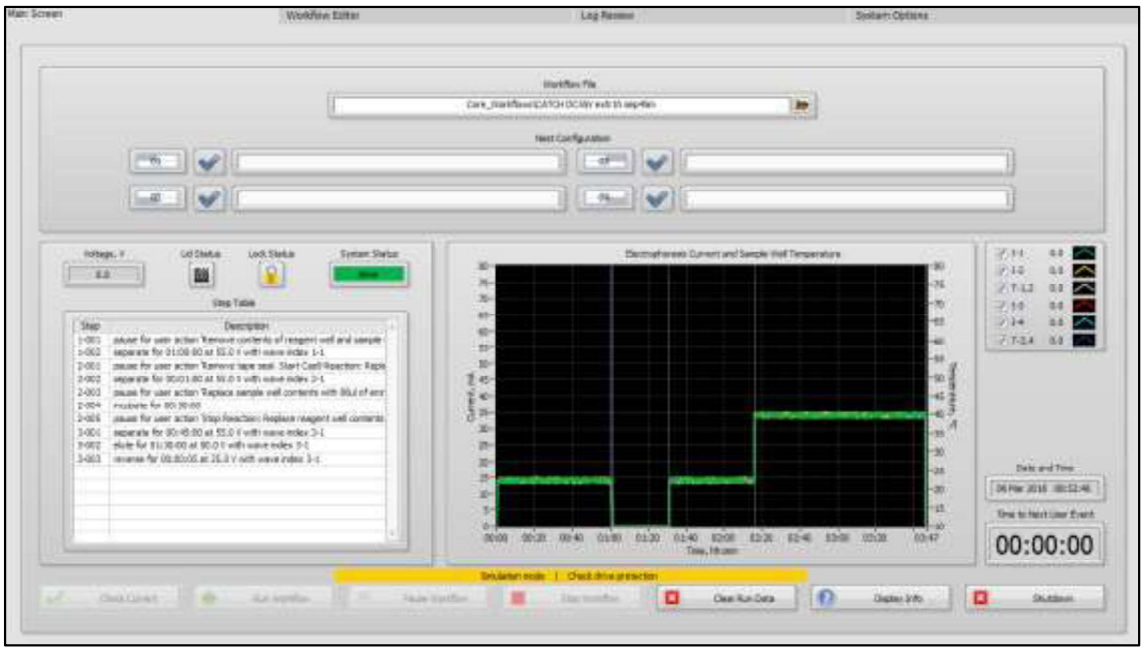
H. Stage 3: Collection Stage

The Collection Stage will require several hours of unattended operation. Users should note the time remaining, after which the samples can be collected.



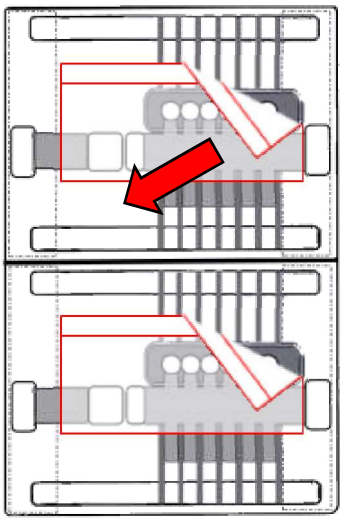
Time remaining to the end of the run

Main Screen at Run Completion:



1. After the run is complete, open the lid and remove the sealing tape from the cassette(s).

grab the tab in right upper corner and peel diagonally with a slow smooth motion



2. Using a wide-bore pipette tip, remove the contents of the elution modules.

Important! 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.

I. Analysis

To identify the position of CATCH targets in the elution products, following procedures are recommended:

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

1. Qubit Assay

The Qubit assay will give a semi-quantitative evaluation of the quality of the CATCH procedure. Extremely HMW DNA will be very inhomogeneously distributed in the elution product. To quantify, we recommend Qubit assays using at least three 1 ul aliquots from different locations within the tube. Average the three readings. A high average value with a high CV is diagnostic of very HMW DNA.

For Qubit assays, using at least three 1 ul aliquots from different locations within the tube

The expected quantity of DNA detected can depend on the Stage 1 extraction method:

1 hour extraction: ~10ng DNA in each elution well. This is indicative of the presence of non-target DNA, which is beneficial as genomic reference for **10X Genomics linked-read analysis.**

3 hour extraction: ~2ng DNA in each elution well, with up to 20ng in non-target wells (#5 or 6) only under some separation conditions. These more highly purified samples are better suited for **PacBio or Oxford Nanopore sequencing.**

2. qPCR Assay

If possible “custom” Taqman qPCR assays from Thermo Life Technologies are recommended for target detection. SYBR green kits may be used for regions outside of genes.

Based on an input of 300,000-400,000 diploid mammalian cells:

1 hour extraction: For single-plex CATCH target enrichment factors of 15-50x relative to a single copy reference gene (RNaseP RNA gene) is typical.

3 hour extraction: Enrichment relative to a reference gene can be as high as 700-fold.