Journal of Visualized Experiments

Automating a positron-emission tomography (PET) radiotracer synthesis protocol for clinical production

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE58428R2
Full Title:	Automating a positron-emission tomography (PET) radiotracer synthesis protocol for clinical production
Keywords:	Positron-emission tomography; automated radiosynthesis; clinical validation; ELIXYS; Clofarabine; clinical utility; radiotracer
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Open Access (US$4,200)
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TITLE:

Automation of a Positron-emission Tomography (PET) Radiotracer Synthesis Protocol for Clinical Production

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KEYWORDS:

Positron-emission tomography, automated radiosynthesis, clinical validation, ELIXYS, Clofarabine, clinical utility, radiotracer

SHORT ABSTRACT:

Positron-emission tomography (PET) imaging sites that are involved in multiple early clinical research trials need robust and versatile radiotracer manufacturing capabilities. Using the radiotracer [^{18}F]Clofarabine as an example, we illustrate how to automate the synthesis of a radiotracer using a flexible, cassette-based radiosynthesizer and validate the synthesis for clinical use.

LONG ABSTRACT:

The development of new positron-emission tomography (PET) tracers is enabling researchers and clinicians to image an increasingly wide array of biological targets and processes. However, the increasing number of different tracers creates challenges for their production at radiopharmacies. While historically it has been practical to dedicate a custom-configured radiosynthesizer and hot cell for the repeated production of each individual tracer, it is becoming necessary to change this workflow. Recent commercial radiosynthesizers based on disposable cassettes/kits for each tracer simplify the production of multiple tracers with one set of

equipment by eliminating the need for custom tracer-specific modifications. Furthermore, some of these radiosynthesizers enable the operator to develop and optimize their own synthesis protocols in addition to purchasing commercially-available kits. In this protocol, we describe the general procedure for how the manual synthesis of a new PET tracer can be automated on one of these radiosynthesizers and validated for the production of clinical-grade tracers. As an example, we use the ELIXYS radiosynthesizer, a flexible cassette-based radiochemistry tool that can support both PET tracer development efforts, as well as routine clinical probe manufacturing on the same system, to produce [^{18}F]Clofarabine ([^{18}F]CFA), a PET tracer to measure *in vivo* deoxycytidine kinase (dCK) enzyme activity. Translating a manual synthesis involves breaking down the synthetic protocol into basic radiochemistry processes that are then translated into intuitive chemistry "unit operations" supported by the synthesizer software. These operations can then rapidly be converted into an automated synthesis program by assembling them using the drag-and-drop interface. After basic testing, the synthesis and purification procedure may require optimization to achieve the desired yield and purity. Once the desired performance is achieved, a validation of the synthesis is carried out to determine its suitability for the production of the radiotracer for clinical use.

INTRODUCTION:

An increasing array of biological targets can be dynamically visualized in living subjects *via* the molecular imaging modality PET. PET provides *in vivo* assays of specific biological, biochemical, and pharmacological processes by using specific radiotracers (molecules labeled with positron-emitting radionuclides) that are injected into the subject prior to imaging[1]. The increased use of PET to study a wide variety of these processes in basic science and clinical research[2-4], and in the discovery, development, and clinical use of drugs in patient care[5,6], is leading to a growing demand for diverse radiotracers[7,8]. To avoid radiation exposure to the radiochemist and to ensure a reproducible production of these short-lived tracers, they are typically manufactured using an automated radiosynthesizer operating inside a "hot cell". Recent radiosynthesizers use a disposable-cassette/kit architecture to simplify the task of complying with clinical-grade manufacturing while also providing the flexibility to prepare multiple types of radiotracers simply by swapping out cassettes[9]. However, in early clinical stages, there are usually no commercially-available cassettes/kits to perform the automated radiosynthesis; consequently, PET drug manufacturing facilities struggle to customize systems to implement cGMP-grade tracer production capabilities within a suitable timeframe and at a reasonable cost. Thus, radiosynthesizers have been developed that combine the cassette/kit architecture with features to facilitate the development and optimization of tracers.

The ELIXYS FLEX/CHEM (ELIXYS) is an example of a flexible cassette-based radiosynthesizer with a wide reagent, solvent, and reaction temperature compatibility[10]. It has three reaction vessels and uses a robotic mechanism to dynamically configure the fluid pathway as required by any particular synthesis protocol[11]. The synthesizer software allows the creation of synthesis programs (**Sequences**) for different tracers by dragging and dropping **Unit Operations** such as **Trap Isotope**, **Elute Isotope**, **Add Reagent**, **React**, and **Evaporate**[12]. Each unit operation has a variety of programmable parameters available to the operator, such as **Duration**, **Temperature**, or inert gas driving pressure (**Pressure**). By understanding the nature of each unit operation, a

manual synthesis can be readily translated into a sequence of unit operations and then be modified during the optimization of the protocol[13]. In combination with the ELIXYS PURE/FORM module, the integrated system can also perform an automated purification and formulation of the PET tracer. Using this radiosynthesizer, we have previously reported the automated synthesis of 24 different ^{18}F-labeled tracers and prosthetic groups[11,14-16], as well as the automated enzymatic radiofluorination of biomolecules[17], by simply changing reagents and not the configuration of the system. Others have shown the automated synthesis of [^{18}F]RO6958948 for the imaging of tau neurofibrillary tangles[18], the automated synthesis of the prosthetic group [^{18}F]F-Py-TFP with a subsequent labeling of peptides[19], and the automated synthesis of [^{18}F]AM580 for the imaging of phosphodiesterase 10a (PDE10A)[20]. Furthermore, several groups have shown the production of tracers suitable for clinical use, including 4-[^{18}F]Fluorobenzyl-triphenylphosphonium ([^{18}F]FBnTP) for the imaging of mitochondrial membrane potential[21], [^{18}F]DCFPyL for the imaging of prostate-specific membrane antigen (PSMA)[22], and [^{18}F]THK-5351 for the imaging of tau[23].

In this paper, we use our experience with [^{18}F]CFA to illustrate how a manual radiosynthetic procedure can be straightforwardly and rapidly translated into an automated synthesis suitable for routine production following cGMP guidelines. The tracer [^{18}F]CFA was designed for the imaging of dCK activity. The manual radiosynthesis of [^{18}F]CFA was originally described by Shu *et al.*[24] as a procedure using two reaction vessels, intermediate silica cartridge purification, and a final HPLC purification step (see **Supplementary Material**, **Section 1** for details). Recent *in vitro* and preclinical studies have shown the exceptional specificity of this tracer to dCK, and first-in-human studies have shown favorable biodistribution[25]. There is an immediate interest in wider-scale clinical studies to confirm the sensitivity of [^{18}F]CFA PET to variations in dCK activity and a longer-term interest in the potential clinical applications of this tracer[26]. It may be a useful biomarker for therapies that trigger T-cell activation, induce DNA damage, or rely on dCK-dependent nucleoside analog prodrugs. In particular, [^{18}F]CFA may enable the stratification of patients for a potential response to treatment with Clofarabine. [^{18}F]CFA may also facilitate the study and development of dCK inhibitors that are advancing toward clinical trials. Since this tracer has traditionally been synthesized manually, advancing all of these studies requires a reliable, automated synthesis of [^{18}F]CFA suitable for clinical use.

Although we previously reported an automated synthesis of [^{18}F]CFA for preclinical studies[16], this protocol builds further on these efforts and describes additional modifications needed for the clinical production of this tracer, including the integration of fully-automated purification and formulation, protocol validation, and quality-control testing. The general procedures described here are not limited to developing an automated and clinically-suitable synthesis of [^{18}F]CFA but can be generalized in a straightforward manner to develop automated syntheses suitable for clinical use of other radiotracers labeled with fluorine-18.

PROTOCOL:

1. General Procedure for the Automation and Validation of a Radiosynthesis Protocol for Clinical Manufacturing

1.1. Analyze the eligibility of the manual synthesis scheme for clinical manufacturing

1.1.1. Perform risk analysis of product contamination with any undesired residual chemicals.

1.1.1.1. Avoid Class 1 solvents such as benzene and replace them with appropriate alternative solvents (Class 2 or Class 3).

1.1.1.2. Avoid chemicals that would be difficult to detect in the final formulation as potential residual impurities.

1.1.1.3. Choose only chemicals that are commercially available in high purity grade (the USP or Ph.Eur. grade desired) and are provided with a certificate of analysis.

1.1.2. Refine the synthesis scheme if any undesirable chemicals or solvents are detected by the risk analysis and repeat section 1.1 until none remain.

1.2. Automate the synthesis protocol

1.2.1. If an automated protocol for the tracer using the same synthesizer has already been created and uploaded to an online repository, download a copy of the synthesis program.

1.2.2. If an automated synthesis program does not already exist, create one.

1.2.2.1. Using paper and pen, divide the manual synthesis into high-level steps (*e.g.*, drying/activating [^{18}F]fluoride, heating to facilitate a radiochemical reaction, performing a purification step, *etc.*). Further break down the high-level steps into discrete, basic processes that are required. As an example, the synthesis scheme of [^{18}F]CFA is shown in **Figure 1**, the identification of high-level steps is shown in **Figure 2A**, and the breakdown into processes is shown in **Figure 2B**.

1.2.2.2. Using paper and pen, map each process into the individual unit operations provided by the synthesizer software. As an example, an analysis of the mapping of basic processes in the synthesis of [^{18}F]CFA to suitable unit operations in the synthesizer software[13] is shown in **Figure 2C**.

1.2.2.3. Using the radiosynthesizer programming interface, create a blank program and append each of the identified unit operations in sequence by clicking the **Menu** button (top left) and selecting **Sequences**, and then clicking the **New Sequence** button. For each unit operation identified in step 1.2.2.2, drag the unit operation from the available operations to the filmstrip view and click or type to fill out the desired value of each parameter of the unit operation. **Figure 3** shows an example of the interface when all the operations to synthesize [^{18}F]CFA have been populated, and the user has selected the first **REACT** unit operation to edit parameter values. The final synthesis program for [^{18}F]CFA is described in the **Supplementary Material**, **Tables S1**

and **S2**.

1.2.3. Verify the synthesis program.

1.2.3.1. Perform a dry run. Set up and run the program as in steps 2.1 - 2.3, using all reagents and solvents other than the radionuclide (*e.g.*, [^{18}F]fluoride) to verify expected behavior.

1.2.3.2. If necessary, adjust the unit operation parameter values in the program (*e.g.*, the time or driving pressure to completely transfer a reagent, the time/temperature to evaporate a solvent to the desired level, *etc.*), and retest. To adjust parameter values, first, return to the **Edit** mode by choosing **Sequences** from the main menu (top left) and select the newly created program. Next, click on the desired unit operation in the filmstrip view (bottom of the screen), navigate to the desired parameter, and select or type the new value.

1.2.4. Perform a low-activity (< 370 MBq) test run to evaluate the program.

1.2.4.1. Optimize the automated synthesis by adjusting parameter values to improve the yield, synthesis time, repeatability, and any other desired measurable outcome.

1.3. Develop quality control (QC) testing procedures

1.3.1. Using a non-radioactive reference of the final product and samples of potential chemical impurities, develop an analytical radio-HPLC and/or radio-thin layer chromatography (radio-TLC) method with sufficient separation of species for the determination of chemical purity, molar activity, radiochemical purity, and radiochemical identity. Validate the analytical method(s) for repeatability and linearity and determine the detection and quantification limits.

1.3.2. Similarly, develop and validate a gas chromatography method to analyze volatile impurities (*e.g.*, residual amounts of solvents that are used during the synthesis).

1.3.3. Develop and validate analytical assays that allow the detection and quantification of other potential impurities (*e.g.*, cryptand 222 *via* the standard color spot test).

1.3.4. Use standard procedures for the determination of sterility, pH, radionuclidic identity, radionuclidic purity, radioactivity concentration, product volume, and endotoxin levels.

1.4. Perform synthesis validation

1.4.1. Establish standard operating procedures (SOPs) for the synthesis and QC testing procedures and integrate a materials and equipment tracking system compliant with current good manufacturing practice (cGMP) requirements.

1.4.2. Validate the synthesis procedures *via* three independent and consecutive production runs at the same radioactivity levels as intended for clinical manufacturing following the SOPs.

Document the synthesis performance and results of QC testing.

1.4.3. All consecutive validation runs must pass the pre-set QC limits. If a validation run fails, repeat the whole validation process after appropriately addressing the root cause of the failure.

2. Example: Automated Synthesis of [^{18}F]CFA for Clinical Use

2.1. Prepare the radiosynthesizer

2.1.1. Power on the radiosynthesizer.

2.1.2. Ensure the inert gas supply is turned on with sufficient pressure and that the necessary valves are open so that the radiosynthesizer is connected to the gas supply.

2.1.3. Install new disposable cassettes in Reactor #1 and #2 positions and insert reaction vessels containing magnetic stir bars. Ensure that each cassette transfer dip tube is pointed straight down.

2.1.4. Prepare reagent vials and install them in the cassettes according to the diagram in **Figure 4**.

2.1.5. Install an empty [^{18}O]H_2O recovery vial in the **W1** position of Cassette #1.

2.1.6. Activate a quaternary methylammonium (QMA) cartridge by first passing 12 mL of a 1 M $KHCO_3$ solution through it, followed by 12 mL of deionized water. Condition a silica Sep-Pak cartridge by passing 5 mL of ethyl acetate through it.

2.1.7. Connect the cartridges and make all cassette tubing connections as shown in **Figure 5A**. Verify that no cassette tubing (including unused tubing) hangs in the interior, where it may interfere with robotic movements.

2.1.8. Connect the [^{18}F]fluoride source line from the cyclotron to the [^{18}F]fluoride input line on Cassette #1.

2.1.9. Ensure the waste container is empty. Place waste lines from the purification/formulation subsystem to the waste container (*i.e.*, sample loop 1 waste line, HPLC subsystem waste line, and the syringe pump waste line).

2.1.10. Connect the HPLC input lines. Place HPLC mobile phase input line “A” in a container of 25 mM ammonium acetate and HPLC mobile phase input line “B” in a container of EtOH.

2.1.11. Equilibrate the purification/formulation subsystem and HPLC column.

2.1.11.1. Open the control page for the purification/formulation module in the software by

selecting **HPLC** from the main menu (top left). By default, the **Purification** tab will already be selected. (This page is shown in **Figure 6.**)

2.1.11.2. Set the flow rate to 5.0 mL/min at the defined solvent composition and choose which column position the purification column is installed in. Turn on the HPLC pump in the isocratic mode for at least 10 min.

2.1.11.3. Rinse the product line and all fraction collection lines with the mobile phase, each for 1 min.

2.1.11.4. Rinse each HPLC sample loop and HPLC sample loop transfer tubing with 10 mL of the mobile phase using a syringe.

2.1.12. Connect the purification/formulation subsystem syringe pump input lines. Use concentrated sodium chloride (90 mg/mL) for the **Elute** line and 0.9% saline for the **Reconstitute** line.

2.1.13. Prime the formulation subsystem.

2.1.13.1. Navigate to the **Formulation** tab of the purification/formulation control page.

2.1.13.2. To prime the concentrated sodium chloride (90 mg/mL), select the **Elute** tab. Press **Initialize** to initialize the syringe pump. Dispense 5 mL.

2.1.13.3. To prime the 0.9% saline, select the **Reconstitute** tab. Dispense 5 mL.

2.1.14. Connect the **Product** and **Final product** lines from the front of the purification/formulation subsystem in a T-connection. Connect the output of the T-connection to a sterile filter (0.22 µm) which, in turn, is connected to the final sterile product vial. Insert a vent needle with a sterile filter into the headspace of the final product vial. A photograph of the final system set-up is shown in **Figure 5B**.

2.1.15. Add dry ice and EtOH or MeOH to the cold trap.

2.2. Run the synthesis program

2.2.1. Navigate to the list of programs by selecting **Sequences** from the main menu button (top left). Select the [^{18}F]CFA program and start the program by pressing the **Run** button.

2.2.2. Carefully go through each item on the pre-run checklist and check them off as they are completed. Part of the pre-run checklist screen is shown in **Figure 7**.

2.2.3. Press **Continue** to confirm the setup is complete and cause the automated synthesis to

begin.

2.2.3.1. If desired, monitor the synthesis in real-time *via* visual feedback (reactor cameras), sensor readings (*e.g.*, temperature, pressure, vacuum, radiation reading, *etc.*) and countdown timers. A representative screenshot is shown in **Figure 8**.

2.2.3.2. During the **Purification** unit operation, select **Product** when the product peak has begun to appear on the radiation detector chromatogram. A representative screenshot during this unit operation (containing a chromatogram of the UV detector and radiation detector output) is shown in **Figure 9**.

2.2.3.3. Once the radiation detector chromatogram peak has returned to the baseline, select **Waste** to divert the flow path of the HPLC subsystem to the waste container.

2.3. Set up and run the Formulation program

2.3.1. From the list of programs (**Sequence** screen), open the **[^{18}F]CFA Formulation** program.

2.3.2. Adjust the parameters of the formulation unit operation.

2.3.2.1. Calculate the volume of the collected product fraction ($V_{fraction}$) based on the HPLC pump flow rate and the duration of the fraction collection.

2.3.2.2. Calculate the volume of additional sodium chloride (90 mg/mL) required to achieve isotonicity and calculate the amount of additional saline required to dilute the EtOH concentration below 10%.

2.3.2.3. Modify the program with these values. The volume of sodium chloride (90 mg/mL) is entered for the **Elute** step and the volume of saline is entered for the **Reconstitute** step. (The calculations are described in the **Supplementary Material**, **Figure S2**.)

2.3.2.4. Save the program.

2.3.3. Run the program. The system will dilute the collected purified product fraction with sodium chloride and saline to ensure the isotonicity of the formulation and deliver it through a sterilizing filter into the sterile product vial.

2.4. Collect formulated [^{18}F]CFA for quality control and shipment

2.4.1. Remove the formulated [^{18}F]CFA product from the hot cell.

2.4.2. Using sterile working techniques, withdraw two samples (300 µL) to perform quality control tests.

2.4.3. Use the first sample to test for sterility of the final formulation by inoculating fluid thioglycolate media and tryptic soy broth for 14 d without observing any growth.

2.4.4. Use the second sample to perform quality control according to the procedures developed in step 1.3. The procedures established at the UCLA Ahmanson Biomedical Cyclotron Facility in accordance with the U.S. Pharmacopeia are described below.

2.4.4.1. Assess appearance by visual inspection.

2.4.4.2. Assess pH with an indicator paper.

2.4.4.3. Assess bacterial endotoxin content using a kinetic chromogenic Bacterial Endotoxin Test (BET).

2.4.4.4. Assess radiochemical identity with analytical radio-HPLC by verifying the co-elution of the radioactive sample and a non-radioactive reference compound.

2.4.4.5. Assess radiochemical purity with analytical radio-HPLC by comparing the area under the curve (AUC) of radioactive impurities in the gamma-detector chromatogram with the AUC corresponding to the desired product.

2.4.4.6. Assess chemical purity with analytical HPLC by determining the AUC in the UV-detector chromatogram of all UV-active impurities.

2.4.4.7. Assess molar activity and carrier mass with analytical radio-HPLC by determining the AUC corresponding to the desired product in the UV-detector chromatogram.

2.4.4.8. Assess the half-life of the probe by measuring its activity at two different timepoints and fitting a decay curve.

2.4.4.9. Assess the residual solvent content of the formulation by gas chromatography.

2.4.4.10. Assess the radionuclide energy using a gamma spectrometer.

2.4.4.11. Assess the cryptand 222 content using a TLC-based spot test.

2.4.5. If all tests pass, release the probe formulation for shipment to the clinical imaging site.

2.5. Post-run and system shutdown

2.5.1. Rinse the HPLC purification column and all tubing used for product collection with 70% (v/v) EtOH in water. This should be done with the PURE/FORM Control page, similar to step 2.1.12.

2.5.2. Shut down the radiosynthesizer *via* the **Power** button on the software. A pop-up window will indicate when the power to the system can be turned off.

2.5.3. Turn off the compressed air and inert gas supplies by closing the appropriate shutoff valves.

2.5.4. Allow time for residual radioactivity in the hot cell to decay (typically overnight).

2.6. Clean the radiosynthesizer

2.6.1. Remove and dispose of all cassettes, cartridges, reactor vials, and reagent vials used during the synthesis.

2.6.2. Empty the contents of the cold trap.

2.6.3. Clean the purification subsystem fluid paths.

2.6.3.1. Open an existing cleaning program or create a new program which contains one Purification unit operation in cleaning mode (*i.e.*, with the **Clean** checkbox selected). See **Supplementary Material**, **Figure S9** for an example.

2.6.3.2. On the parameter configuration page, select the column that was used for the purification and the HPLC mobile phase input line that is connected to a bottle containing 70% EtOH in water. Program a flow rate of 2 mL/min, a rinsing duration for each injection loop of 5 min, and a rinsing duration for each product and fraction output of 30 s. Select **Dry Lines** and program a duration of 30 s.

2.6.3.3. Place all fraction line outputs in a large waste container.

2.6.3.4. Run the program.

2.6.3.5. After completion, empty the waste container.

2.6.4. Clean the formulation subsystem fluid paths.

2.6.4.1. Open an existing program or create a new program which contains one Formulation unit operation in cleaning mode (*i.e.*, with the **Clean** checkbox selected under the **Clean** tab). See **Supplementary Material**, **Figure S10** for an example.

2.6.4.2. Fill a clean dilution reservoir (at the front of the purification/formulation subsystem) with 100 mL of EtOH.

2.6.4.3. Place the purification/formulation subsystem **Elute** input line in an EtOH reservoir

(containing > 50 mL of EtOH).

2.6.4.4. Place the **Rinse** and **Reconstitute** input lines in a waste container along with the final product output line.

2.6.4.5. Run the program.

2.6.4.6. After completion, empty the waste container.

REPRESENTATIVE RESULTS:

A method to automate the production of [^{18}F]CFA was developed and three validation batches were synthesized. Synthesis, purification, and formulation of [^{18}F]CFA was achieved in 90 ± 5 min (*n* = 3) and the non-decay-corrected radiochemical yield was 8.0 ± 1.4% (*n* = 3). The activity yields of the three runs were 3.24 GBq, 2.83 GBq, and 3.12 GBq, starting from 34.3 GBq, 41.8 GBq, and 41.1 GBq, respectively. The obtained [^{18}F]CFA formulations passed all quality control tests (**Table 1**). The automated protocol is currently being used for the production of clinical-grade [^{18}F]CFA to support clinical trials.

FIGURE AND TABLE LEGENDS:

Table 1: Quality control (QC) test data summary for three validation batches. EOB = end of bombardment; EOS = end of synthesis; ND = not detected.

Figure 1: [^{18}F]CFA radiosynthesis scheme. MMT = Monomethoxytrityl.

Figure 2: Translation of a manual synthesis into an automated sequence of unit operations. (**A**) This panel gives an overview of the high-level steps in the manual synthesis of [^{18}F]CFA. (**B**) This panel shows the basic procedures needed to perform each of the high-level steps. (**C**) Radiosynthesizer-specific unit operations used to perform the basic procedures are shown as cards. Each unit operation has its own set of parameter values (shown as underlined) which are configured through the software. The notation “R1” and “R2” indicate the reaction vessels #1 and #2, respectively. The reagents corresponding to the reagent numbers are identified in **Figure 4**. The series of unit operations is saved as a **Sequence** and executed by the software to perform the automated synthesis.

Figure 3: Screenshot of the radiosynthesizer (ELIXYS) software interface to create a synthesis program. Unit operations are placed in the desired order in the **Filmstrip** using a drag-and-drop interface. In this screenshot, a **React** unit operation is selected, and its editable parameter values are shown in the main part of the screen. In this example, the fluorination reaction will be carried out in reaction vessel #1 (sealed) at 120 °C for 10 min with active stirring. The vessel will be cooled to 35 °C after the reaction time has elapsed. Details of parameter values that can be programmed for other unit operations are shown in the **Supplementary Material**, **Section 3**.

Figure 4: Screenshot of the reagent configuration screen. For the [^{18}F]CFA synthesis sequence,

all reagents are loaded into disposable cassette #1, which is shown highlighted in the component selection area. For the [^{18}F]CFA synthesis described here, **Eluent** is 1.0 mg of K_2CO_3 + 5.0 mg of K222 in 0.4 mL of H_2O/0.5 mL of MeCN, **Precursor** is 6 mg of CFA precursor in 0.6 mL of MeCN, and **HPLC Mobile Phase** is 85:15 v/v 25 mM ammonium acetate:ethanol.

Figure 5: Radiosynthesizer set-up for the synthesis of [^{18}F]CFA. (**A**) This is a schematic showing cassette fluid paths, connections to cartridges, and the connection to transfer final crude product from the radiosynthesis module to the purification/formulation module. (Both modules are controlled with a single computer and software interface.) (**B**) This is a photograph of the radiosynthesizer inside a hot cell after the preparation for [^{18}F]CFA synthesis.

Figure 6: Screenshot of the purification/formulation module control interface. This screen is accessed by the operator to manually control the HPLC and formulation subsystems during the synthesis setup.

Figure 7: Pre-run checklist screen. The operator enters the serial number of the cassettes installed in the system and must check off each item to ensure the system has been properly configured and prepared for the synthesis. In addition to these sections, the operator is also prompted for a name and description of the synthesis run (Section 1) and lot numbers for all reagents used (Section 2) and is asked to verify all reactor video feeds are functioning correctly (Section 6).

Figure 8: Screenshot of the radiosynthesizer software while running the [^{18}F]CFA synthesis sequence. The software displays the order of unit operations in the filmstrip area. Completed operations are greyed out and highlighted in white, the current operation is highlighted in grey, and upcoming operations are shown in dark grey. The center area of the screen shows the status of the active unit operation, including which subcommand is being executed, as well as the current system status (reactor video feeds and sensor data). This particular **React** unit operation is the fluorination reaction. In the **Temp** area, the current temperature of the reactor is shown next to the target (programmed) temperature. Below this, the **Activity** area displays the radiation sensor values from the three sensors associated with the reaction step. Finally, a video feed on the left shows a live view of the reactor vial.

Figure 9: Screenshot of the radiosynthesizer user interface while running the Purification unit operation during the synthesis of [^{18}F]CFA. The UV detector and radiation detector outputs of the purification/formulation module are displayed on the central graph in real time. Additional feedback from the detectors and HPLC pump are shown on the right side of the screen. The operator collects the product peak by temporarily selecting **Product** when the peak begins to appear and then switching back to **Waste** after the complete peak has been seen.

DISCUSSION:

This protocol defines the basic steps that should be taken when automating a manual synthesis protocol to achieve the production of clinical grade tracer formulation. The entire development cycle, including quality control development, is exemplified by the radiotracer [^{18}F]CFA (for the

imaging of dCK activity). Particular attention was paid to modifying the automated synthesis to ensure the tracer's suitability for clinical use. The synthesis entails basic processes such as the activation of [^{18}F]fluoride, radiofluorination of the precursor molecule, intermediate cartridge purification, protecting-group removal, and semi-preparative HPLC purification and formulation for injection. These basic processes comprise a standard repertoire that is sufficient for the synthesis of the vast majority of ^{18}F-labeled PET tracers.

While designing the synthesis, the choice of reagents and their quality assurance is of particular importance for clinical use. Ensuring the correct programming and proper connections by performing a mock synthesis (solvents only) is imperative to eliminate unexpected errors when the synthesis is performed with radioactivity. The subsequent synthesis optimizations (solvents, volumes, amounts, temperatures, reaction times, and purification conditions) depend on the specific PET tracer in development. During these experiments, particular focus should be shone on the chemical and radiochemical purity of the final product that can be achieved, as these must meet stringent requirements for clinical use. A synthesis that reliably produces a pure product in lower but sufficient activity yields is usually preferred over a higher-yielding process that has a risk to fail sporadically. Once the synthesis has been adequately optimized, the final process needs to undergo validation tests (a regulatory requirement) to ensure clinical suitability. The validated synthesis method can then be used to produce the PET tracer for clinical use. When synthesizing a PET tracer according to a validated method, the standard operating procedures should be thoroughly followed. To ensure compliance, the software is programmed to have the operator confirm the completion of key steps *via* a pre-run checklist after clicking on **Run** to start the synthesis. While the system will perform the synthesis in an automated fashion, the purification step requires manual intervention. The operator must, therefore, closely observe the chromatographic screen during the HPLC purification step, and manually input in real-time when to start and stop collecting the product fraction.

Within our automation and optimization efforts for the [^{18}F]CFA synthesis, we have streamlined the semi-preparative HPLC purification method of the product mixture by using an injectable solvent system consisting of ammonium acetate solution and EtOH; our previous method required an additional step to exchange the solvent after purification[16]. The subsequent formulation process, thus, needs only to reduce the EtOH content of the collected fraction to permitted levels, and to ensure its isotonicity, both of which can be accomplished by dilution. The formulation step was performed using a second program consisting of a single Formulation unit operation to allow variable volume additions of NaCl-solutions to the purified product fraction *via* the formulation module to account for the variable volume obtained after HPLC purification. If the collected product fraction volume was set to be constant instead, the Formulation unit operation could be included in the main synthesis program, avoiding the need for an independent program. An alternative approach to avoid manual intervention would be to use the full functionality of the formulation module (*e.g.*, dilute the purified tracer with water, trap on a C18 solid-phase extraction cartridge, wash it, elute it with a fixed volume of EtOH, and finally, dilute it with a fixed volume of saline).

The technique presented here for automating and validating a synthesis protocol for clinical use

is intended to be quite general. Through the choice of radiosynthesizer (ELIXYS), a wide range of syntheses can be automated and validated. This includes complex 3-pot syntheses, or syntheses involving high temperatures of volatile solvents. Optimizing a synthesis can be achieved by changing the parameters of the software program. The synthesizer has features to monitor the impact of changes, such as positioning the reaction vessels for the removal of samples for radio-TLC or radio-HPLC analysis. However, without system modifications, the system currently does not allow for the handling of very low reagent volumes (~5 - 20 µL), intermediate product distillation, or the handling of [^{18}F]AlF, ^{68}Ga, or other radiometals. If the manual synthesis to be automated contains such steps and they cannot be circumvented, automation and validation with another radiosynthesizer platform may be appropriate.

Although this work has focused on the development of a protocol for the automated production of [^{18}F]CFA for clinical use, the synthesis of many other PET tracers could be automated in a manner suitable for clinical production, following the same logic and methods. Following the method presented here, we have also adapted the automated synthesis of 9-(4-[^{18}F]fluoro-3-[hydroxymethyl]butyl)guanine ([^{18}F]FHBG) and validated it for clinical use. User-established protocols can be uploaded to and downloaded from the SOFIE Probe Network, a web portal for sharing synthesis programs and associated documentation among different radiopharmacy sites[27]. This can avoid a duplication of efforts in the community and facilitate multi-center clinical studies involving PET imaging.

ACKNOWLEDGMENTS:
This work has been supported in part by the National Cancer Institute (R44 CA216539) and the UCLA Foundation from a donation made by Ralph and Marjorie Crump for the UCLA Crump Institute for Molecular Imaging.

DISCLOSURES:
The Regents of the University of California have licensed technology to SOFIE that was invented by Jeffrey Collins and R. Michael van Dam and have taken equity in SOFIE as part of the licensing transaction. Furthermore, R. Michael van Dam is a founder and consultant of SOFIE. The terms of this arrangement have been reviewed and approved by the University of California, Los Angeles in accordance with its conflict of interest policies. Eric Schopf and Christopher Drake are employees and shareholders of SOFIE.

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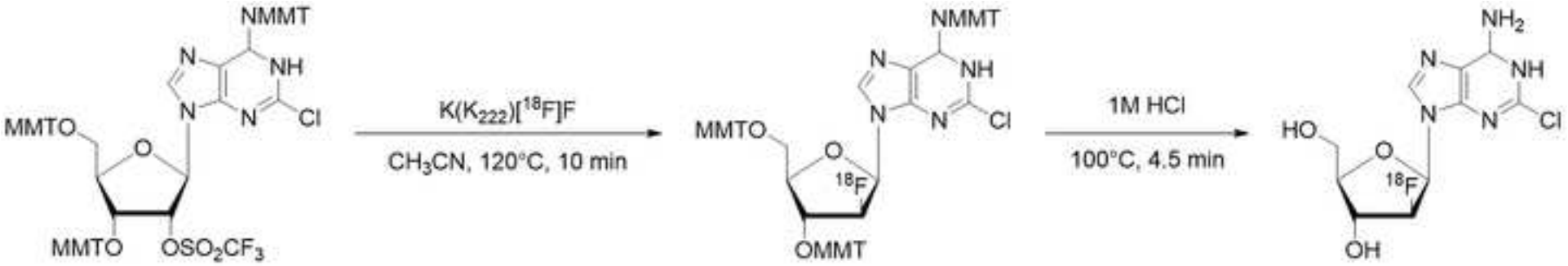

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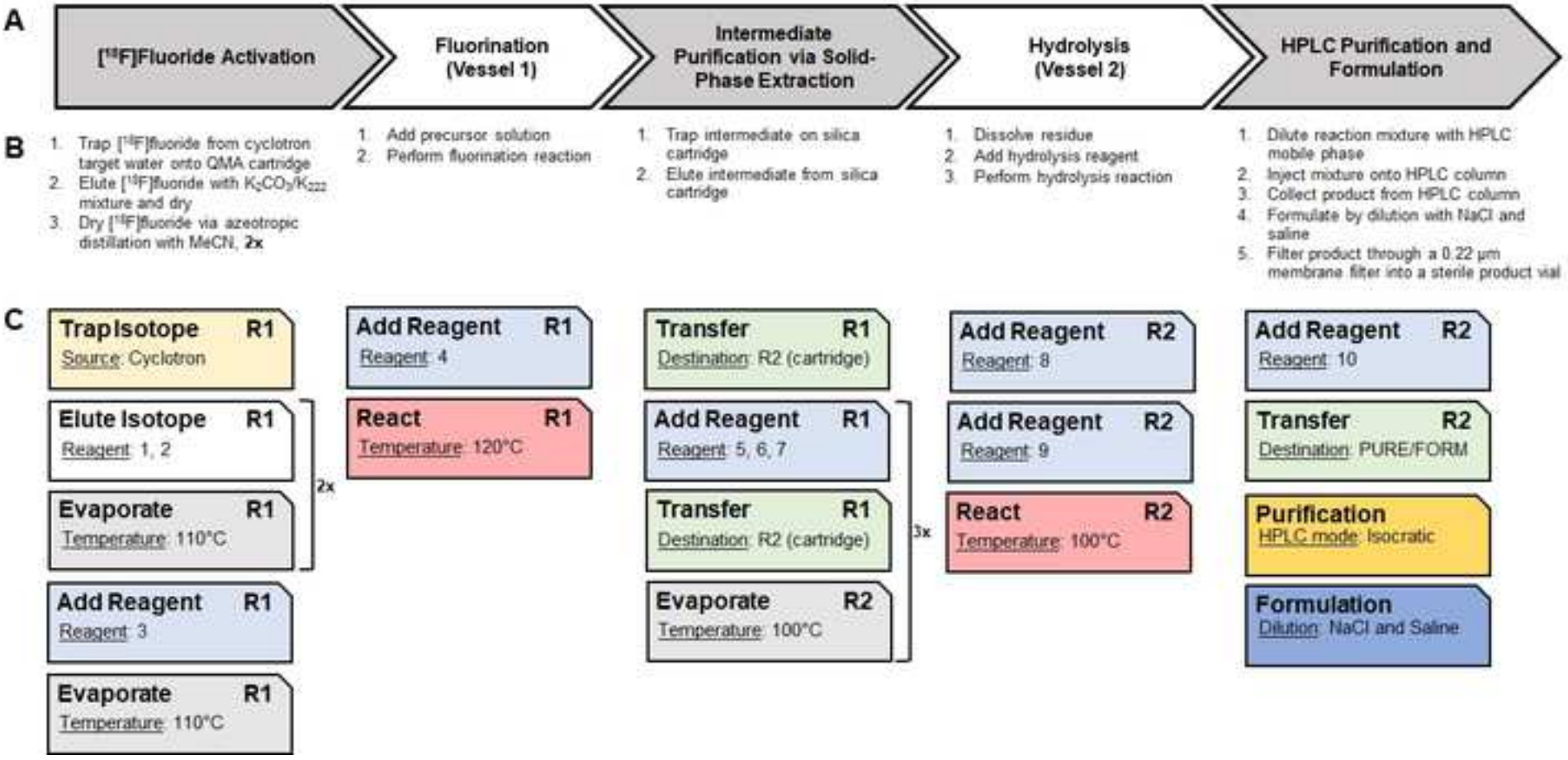

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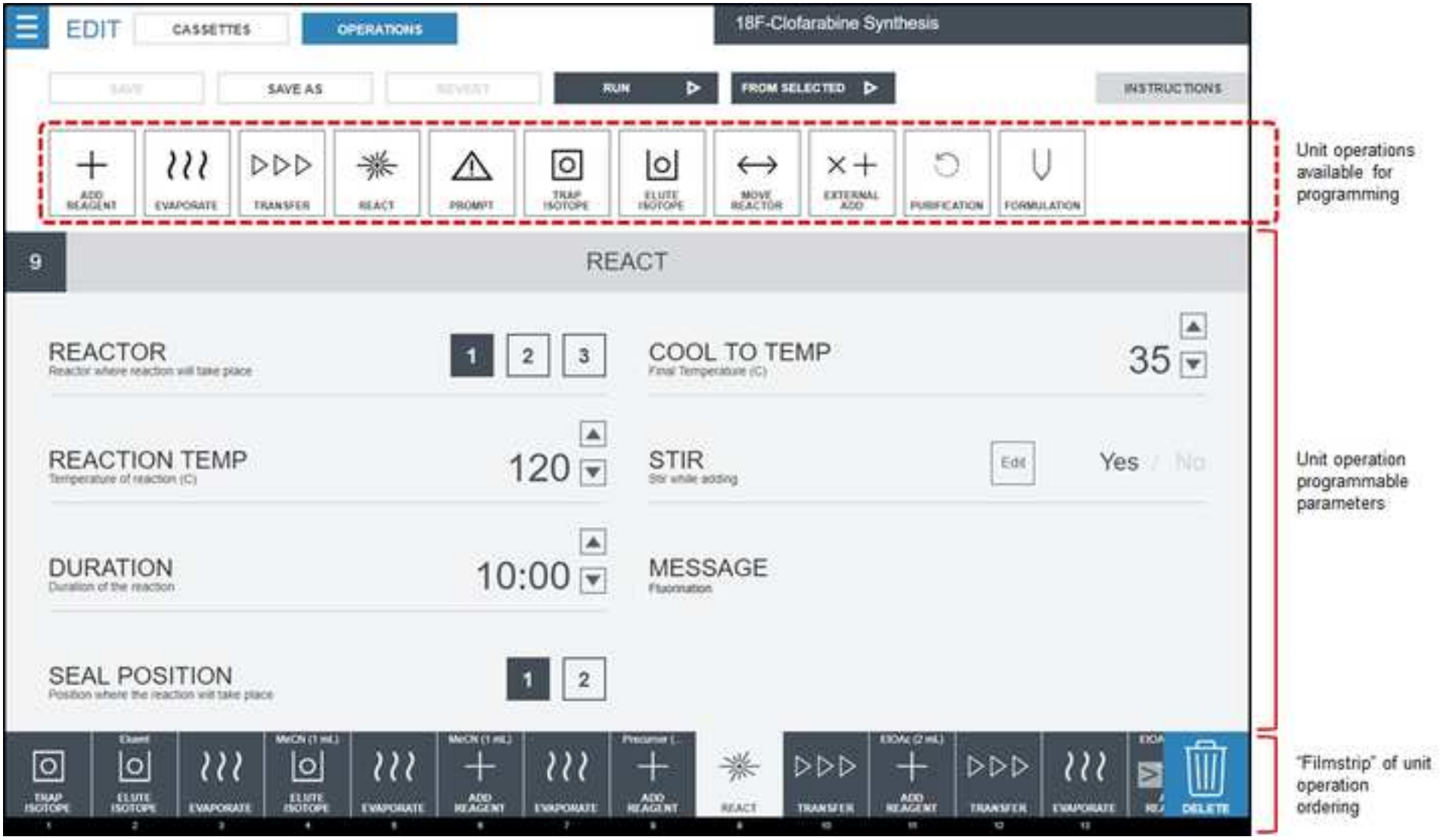

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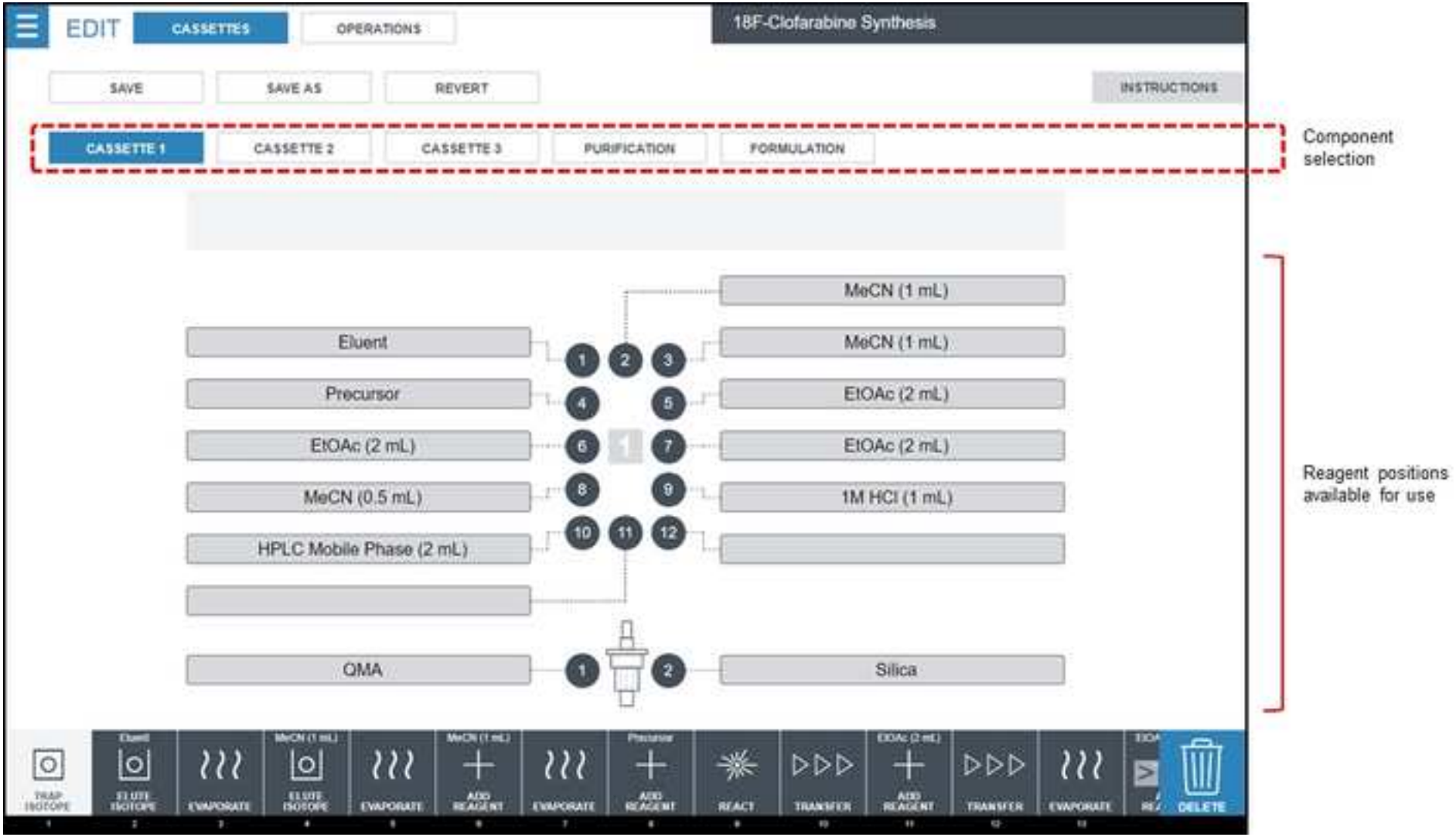

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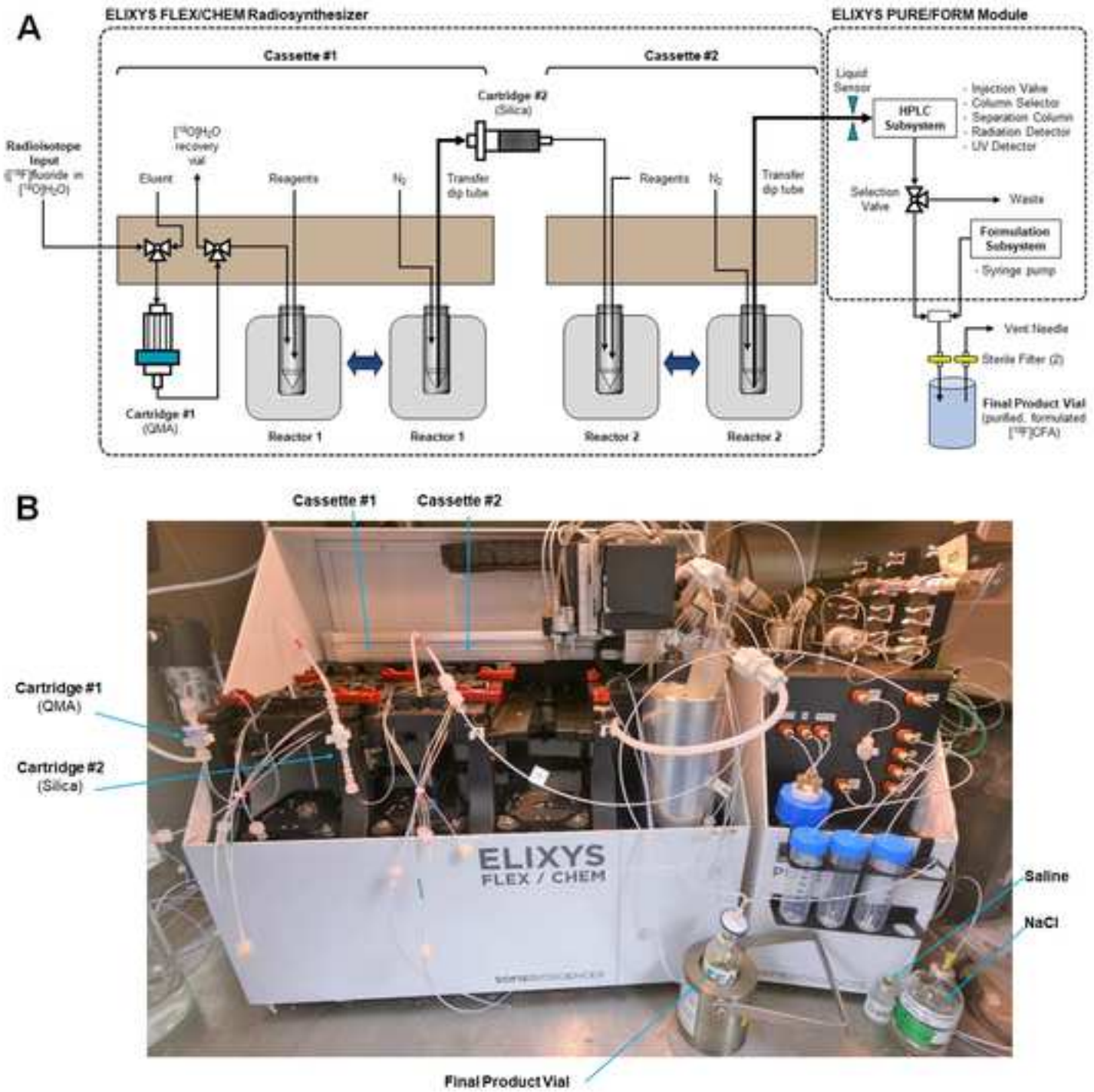

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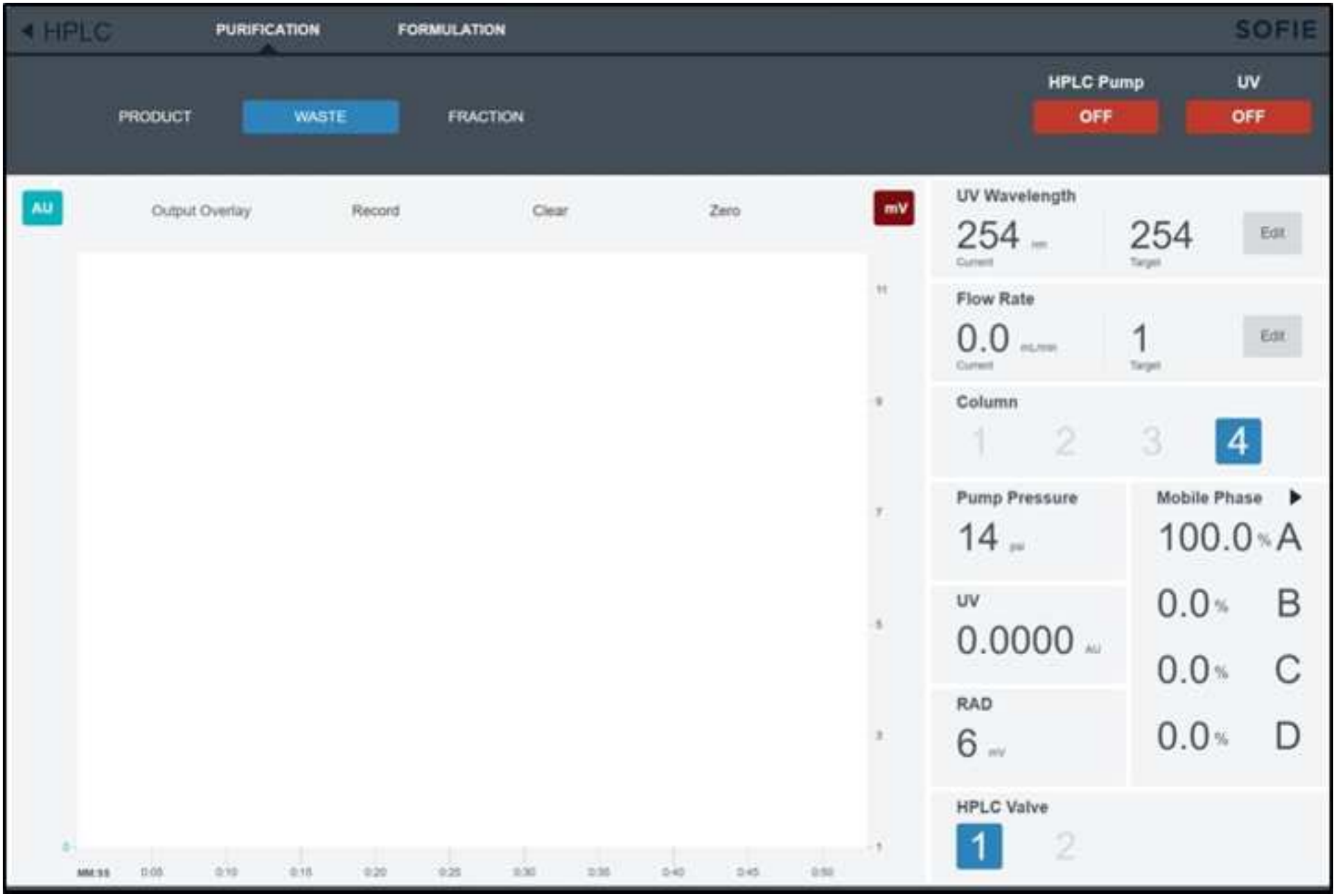

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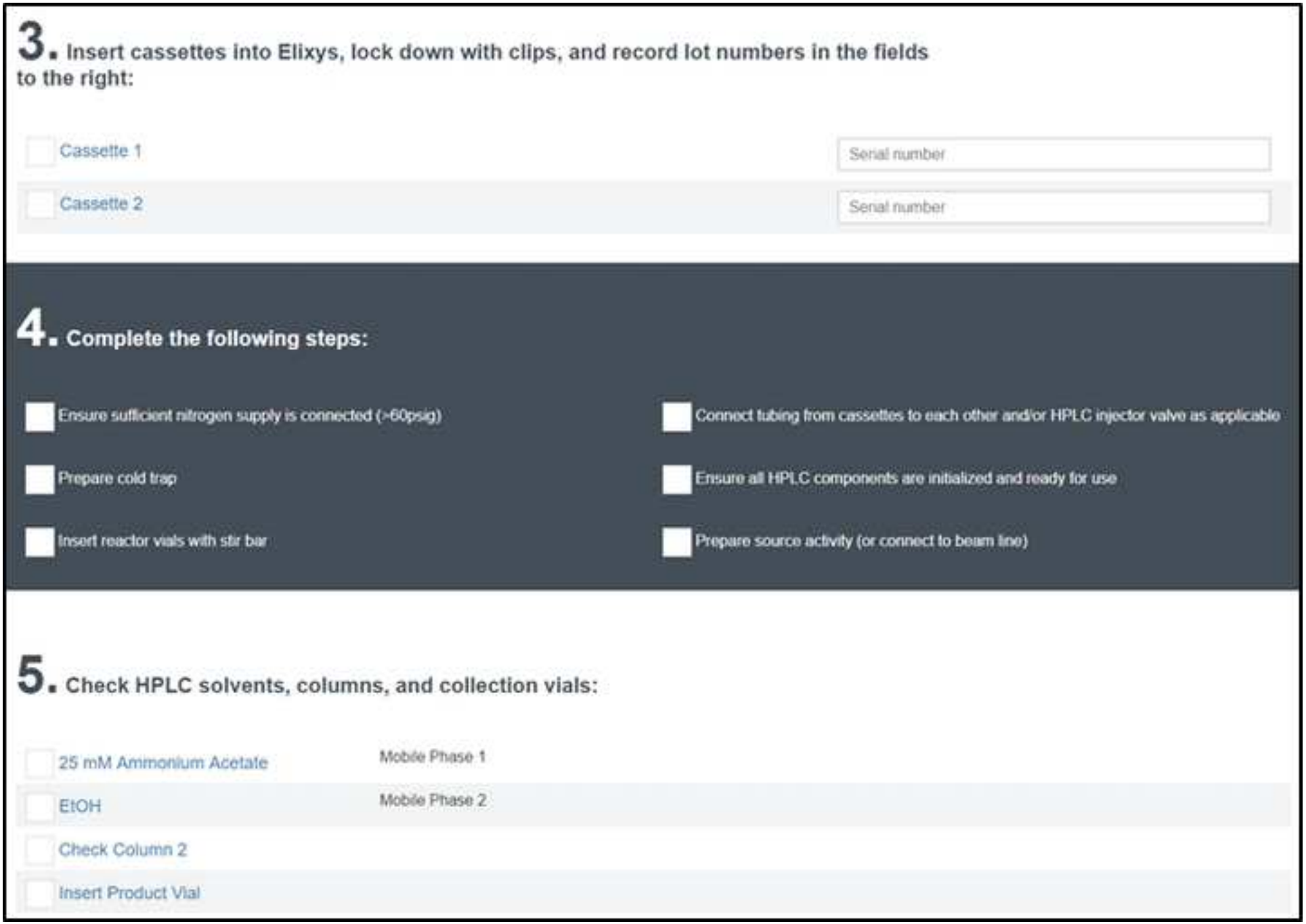

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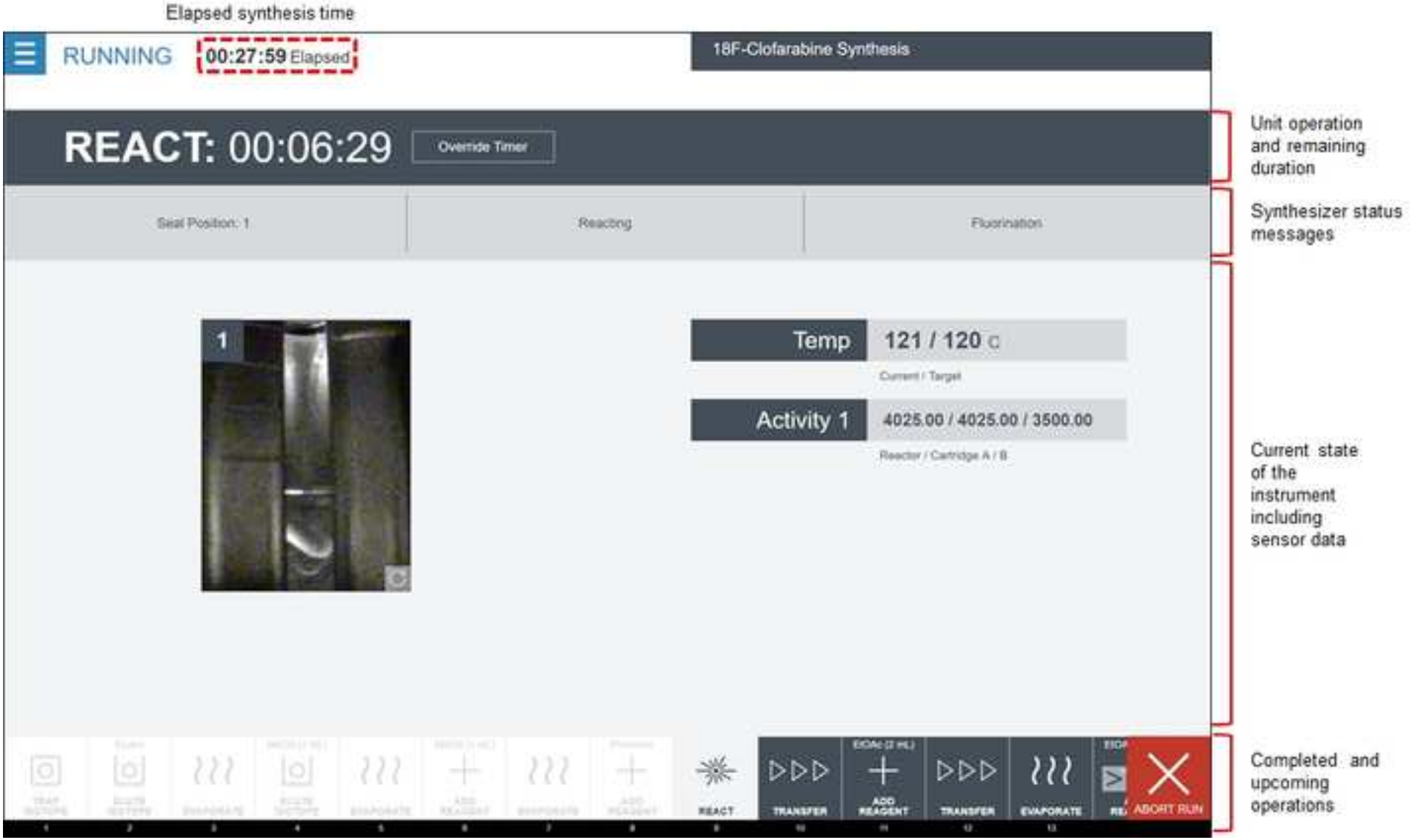

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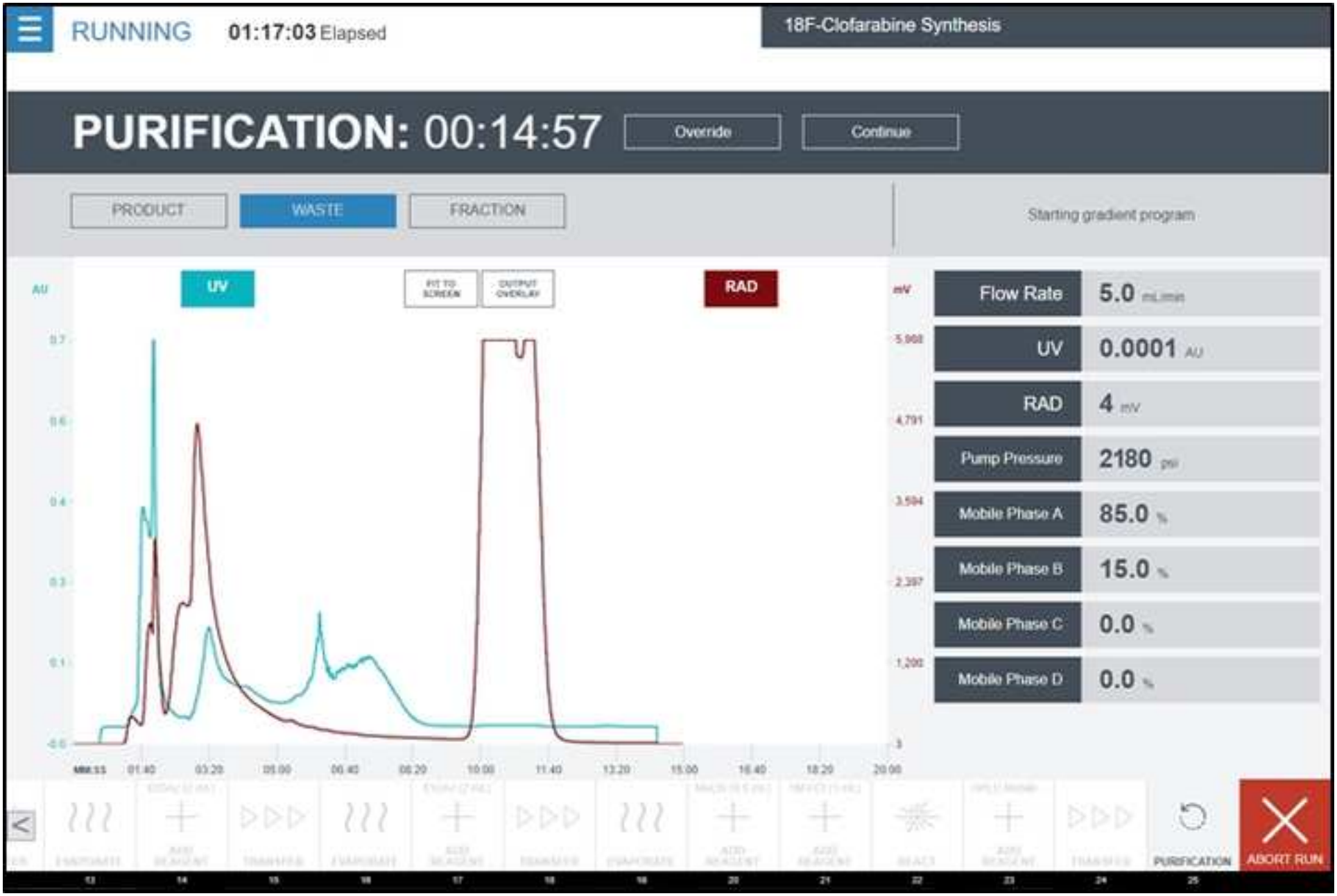

Table 1

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Quality control data [requirement for "Pass"]	Validation run 1	Validation run 2
Appearance [clear, colorless, free of particulate matter]	Pass	Pass
Radioactivity concentration at EOS [≤ 740 MBq/mL @ EOS]	213 MBq/mL	210 MBq/mL
pH [5.0 – 8.0]	6	5.8
Half-life [105 – 115 min]	115 min	108 min
Radiochemical purity [> 95%]	99%	99%
Radiochemical identity by relative retention time (RRT) [1.00 < RRT < 1.10]	1.01	1.01
Molar activity [≥ 3.7 GBq/µmol]	314 GBq/µmol	>370 GBq/µmol
Total carrier mass in final product [≤ 50 µg/dose]	3.1 µg	<1 µg
Total impurity mass in final product [≤ 1 µg / dose]	ND	ND
Maximum allowable injection volume based on total carrier mass ≤ 50 µg/dose AND total impurity mass ≤ 1 µg/dose	Whole batch	Whole batch
Residual EtOH content by GC [≤ 10%]	8.90%	9.50%
Residual EtOAc content by GC [≤ 5000 ppm]	<1 ppm	<1 ppm
Residual MeCN content by GC [≤ 410 ppm]	<1 ppm	<1 ppm
Residual K222 by color spot test [< 50 µg/mL]	Pass	Pass
Filter membrane integrity test [bubble point ≥ 50 psi]	Pass	Pass
Bacterial endotoxins [≤ 175 EU/batch]	Pass	Pass
Radionuclidic purity by gamma spectroscopy [>99.5%]	Pass	Pass
Sterility [meet USP <71> requirements]	Pass	Pass

Validation run 3
Pass
180 MBq/mL
6
112 min
99%
1.01
>370 GBq/µmol
<1 µg
ND

Whole batch

9.60%
<1 ppm
<1 ppm
Pass
Pass
Pass
Pass
Pass

Name of Reagent/ Equipment	Company	Catalog Number
ELIXYS FLEX/CHEM	Sofie (Culver City, CA, USA)	1010001
Radiosynthesizer cassette	Sofie (Culver City, CA, USA)	1861030400
ELIXYS PURE/FORM	Sofie (Culver City, CA, USA)	1510001
[O-18]H2O	IBA RadioPharma Solutions (Reston, VA, USA)	IBA.SP.065
[F-18]fluoride in [O-18]H2O	UCLA	N/A
Deionized water	UCLA	N/A
Acetonitrile (MeCN)	Sigma-Aldrich (St. Louis, MO, USA)	271004
Ethanol (EtOH)	Decon Laboratories, Inc. (King of Prussia, PA, USA)	2701
Sodium hydroxide (NaOH) solution	Merck (Burlington, MA, USA)	1.09137.1000
Hydrochloric acid (HCl) solution	Fisher Chemical (Hampton, NH, USA)	SA48-500
Ethyl acetate (EtAc)	Fisher Chemical (Hampton, NH, USA)	E195SK-4
Sodium chloride (NaCl)	Fisher Chemical (Hampton, NH, USA)	S-640-500
Ammonium acetate	Fisher Chemical (Hampton, NH, USA)	A639-500
Potassium carbonate (K2CO3)	Fisher Chemical (Hampton, NH, USA)	P-208-500
CFA precursor	CalChem Synthesis (San Diego, CA, USA)	N/A
Cryptand 222 (K222; Kryptofix 2.2.2)	ABX Advanced Biochemical Compounds (Radeberg, Germany)	800.1000
Sodium chloride (NaCl) solution (saline)	Hospira (Lake Forest, IL, USA)	0409-4888-02
Silica cartridge	Waters (Milford, MA, USA)	WAT051900

Quaternary methylammonium (QMA) cartridge	Waters (Milford, MA, USA)	WAT023525
Sterile syringe filter (0.22 µm)	Millipore Sigma (Burlington, MA, USA)	SLGSV255F
Glass V-vial (5 mL)	Wheaton (Millville, NJ)	W986259NG
Septa	Wheaton (Millville, NJ)	224100-072
Crimp cap	Wheaton (Millville, NJ)	224177-01
Amber serum vial (2 mL)	Voigt (Lawrence, KS, USA)	62413P-2
Magnetic stir bar	Fisher Scientific (Hampton, NH, USA)	14-513-65

Comments/Description
Radiosynthesizer
Cassette for ELIXYS FLEX/CHEM
Radiosynthesizer purification module
>90% isotopic purity
Produced in a cyclotron (RDS-112; Siemens; Knoxville, TN, USA) by the (p,n) reaction of [O-18]H2O. Bombardment at 11 MeV using a 1 mL tantalum target with havar foil. Purified to 18 MΩ and passed through 0.1 µm filter
Anhydrous, 99.8%
Anhydrous, 200 proof
1M solution
1M solution
HPLC grade
USP grade
HPLC grade
Certified ACS
Custom synthesis
>99%
0.9%, for injection, USP grade
Sep-pak Classic

Sep-pak Light Plus

Millex-GV
Used for reaction vessels
Used for reagent vials
Used for reagent vials
Used for reagent vials
Used for reaction vessels

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Dear Dr. Wu,

Thank you for your careful consideration of our revised manuscript. We have made modifications to the manuscript and supporting information as per your requests. Below is our detailed response to each request. Changes have been tracked in the manuscript and supporting information files.

Editorial comments:

The manuscript has been modified and the updated manuscript, **58428_R1.docx**, is attached and located in your Editorial Manager account. **Please use the updated version to make your revisions.**

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have carefully proofread the manuscript.

2. Please highlight less than 2.75 pages of protocol steps (including spacing and headings) for filming.

We have modified the grouping of a couple of protocol steps to make slight reduction in overall length of highlighted regions. We have checked that the new length is within 2.75 pages (using the current formatting/margins).

3. Please remove the embedded Table from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.

We have moved the table to a separate file, and moved the caption to a 'TABLE LEGENDS' section after the 'REPRESENTATIVE RESULTS'.

4. Author Affiliation: What is SOFIE or UCLA? Please define the abbreviation before use.

SOFIE is not an abbreviation; the company just spells it will all capital letters. We have spelled out the first use of UCLA (University of California, Los Angeles) in the manuscript and supporting information.

5. For steps that are done using software, a step-wise description of software usage must be included in the step. Please mention what button is clicked on in the software, or which menu items need to be selected to perform the step. Please revise the following steps: 1.2.2.1-2.2.4, 1.2.3.1-1.2.3.2, 1.3.1-1.3.3,

We have made the following changes:

For steps 1.2.2.1-1.2.2.3 (1.2.2.1-1.2.2.2 in the new version), we have clarified that these are done with paper and pen, not software.

For step 1.2.2.4 (1.2.2.3 in the new version), it would be very repetitive and lengthy (many additional pages) to write out the full details. For each unit operation identified in Figure 2C, there would be a drag-and-drop operation, then multiple button clicks to set the parameter values for that operation. Programming involves repeating these steps for all unit operations. Will this be okay as is? For the video, we can show in detail how to program the first few of the operations perhaps? This will give the reader enough idea how to perform programming of the entire synthesis (or to generalize it to synthesize other PET tracers). Happy to modify if you have other suggestions here.

For 1.2.3.1, this would require the same steps as 2.1-2.3. I have indicated this in the step.

For 1.2.3.2, we have given more detail how this would be accomplished.

For 1.3.1-1.3.3, these may or may not be performed with software, depending on the particular radio-HPLC and other instruments that the researcher has in their lab. They are complex steps, potentially involving identifying unknown impurities, purchasing or preparing standards for desired compound and impurities, tuning of separation conditions to achieve the desired resolution (developing the method), and then establishing repeatability, linearity, and detection limits (validating the method), and iterating if repeatability, linearity, or detection limit criteria are not met. That being said, the techniques for performing these tasks are well-established in analytical chemistry.

6. 2.4.4: Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?

We have added a lot more detail for this step. We have also elected to unhighlight this section.

7. Please provide a title for the supplementary file.

We have used the title "Supplementary Material". Is this acceptable? We have updated this when uploading the new files.

We thank the reviewers and editor for their time and efforts in reviewing our manuscript. The many helpful questions and suggestions have led to significant improvements in the manuscript. Below, we respond to each individual concern of the editor and reviewers. Original comments are in black text and our responses are in green.

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Checked

2. Figure 1: Please include a space between numbers and its corresponding units (i.e., 120 °C, etc.).

Updated

3. Please provide an email address for each author.

Added to title page.

4. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.

Updated

5. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Kryptofix®222, etc.

Added name Kryptofix 222 to materials table and replaced with more generic name in text. Replaced most instances of ELIXYS and PURE/FORM with generic names throughout text.

6. JoVE policy states that the video narrative is objective and not biased towards a particular product featured in the video. The goal of this policy is to focus on the science rather than to present a technique as an advertisement for a specific item. To this end, we ask that you please reduce the number of instances of "SOFIE", “ELIXYS”, within your text. The terms may be introduced but please use them infrequently and when directly relevant. Otherwise, please refer to the terms using generic language.

Updated

7. 1.3 and 1.4: Unclear what we can show here, please describe the actions. If there are no specific actions being performed I suggest unhighlighting these.

We have elected to remove the highlighting.

8. Please include single-line spaces between all paragraphs, headings, steps, etc.

Added.

9. There is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

We have checked that the length of highlighted region is within limits.

10. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

We have checked that the highlighted steps in sequence form a cohesive narrative.
We have verified that we have always highlighted complete sentences.
We have verified and made changes so that the highlighted part includes at least one action written in the imperative tense.

11. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

We have corrected our highlighting such that the substeps are also highlighted if they provide the detail of how to perform the ‘parent’ step

12. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

We have modified and reorganized the discussion to cover all of the points listed above.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
The presented manuscript accurately presents the issue and the descriptions of each subsequent step are accurate. This manuscript may be presented as a fully sufficient guideline used in the implementation of newly developed radiotracers with keep of all the rules regarding their clinical use.

Major Concerns:
None

Minor Concerns:
None

We thank the reviewer for the positive assessment and remarks.

Reviewer #2:

Manuscript Summary: This manuscript describes an automated radiosynthesis protocol for [18F]Clofarabine including computer-controlled synthesis sequence (labeling reaction, purification and formulation) as well as quality control (QC) procedures. The manuscript is well written and suitable for publication in JoVE.

Minor Concerns:
Comments:
1. The Radiosynthesizer and PURE/FORM Module are united together as a single module with a single computer-controlled system, or two different modules with two computer-controlled systems?

We agree that in the current version the system description was confusing. Indeed, both the synthesizer and purification/formulation module are united together and operated as a single module with a single computer-controlled system. This has been clarified in the manuscript.

2. In the text, RESULTS section, it is better to give molar activity (MA, formerly known as specific activity, SA) data, because MA will significantly affect the clinical evaluation of an F-18 PET tracer. This reviewer noted that SA did appear in QC data table. Also, please change SA to MA, change Ci to GBq (SI unit).

We thank the reviewer for pointing this out. We have corrected specific activity in the results summary table for molar activity. We have further changed all radioactivity units to SI units, from Curie to Becquerel.

3. There are reports to indicate that QMA cartridge might decrease the MA (SA) of F-18 tracers. In ELIXYS module(s), is it able to eliminate the use of QMA cartridge?

We agree that the use of a trap and release cartridge can introduce cold fluoride that subsequently lowers the molar activity of the final product, either by contamination of the exchange resin, the solutions used for cartridge conditioning or the release solution. It is possible to omit the trap and release process of the F-18 ion, which could potentially further increase the molar activity. However, due to the larger amount of water, this may extend the time needed for the [^{18}F]fluoride drying process. Furthermore, the use of the QMA allows recovery of O-18 enriched water for recycling and also has the effect of removing long-lived metal impurity radioisotopes (to avoid interference with reactions and contamination of final product), both of which would not be possible if the QMA is avoided.

4. Why in ELIXYS Radiosynthesizer huge amount of precursor was used in the F-18 radiosynthesis, here 6 mg? The big amount of the precursor can increase the radiochemical yield, but may decrease the MA/SA and chemical purity.

We have not discussed specific synthesis optimization details in this work as our goal was to have a generally applicable process description that fits most tracer productions. In the literature, many tracers are made with several mg of precursor up to 10s of mg of precursor. In this specific case of [^{18}F]CFA, the established manual synthesis actually originally used 10mg of precursor dissolved in 1mL of acetonitrile. Further optimization studies have shown that the precursor concentration is important for high yields, but not the absolute amounts of precursor; thus precursor can be reduced by reducing the reaction volume. Though one of the drawbacks of many macroscale radiosynthesizers, including the ELIXYS system, is the difficulty of using very low volumes, we have been able to reduce the reaction scale to 600 µL (6mg of precursor) while still achieving reliable and robust results. These conditions resulted in a MA of ~350 GBq/µmol and an excellent chemical purity (no impurities detected) as described in the quality control data table.

5. There are some format issue in REFERENCES section, such as superscript issue of 18F.

We thank the reviewer for noticing some errors in the reference sections. We have revised this section and corrected all superscripts.

6. The protocol need to be further polished, because it includes numerous development, optimization and validation runs.

We thank the reviewer for this valuable input. We have thoroughly reconsidered our approaches, however in our opinion the protocol in its original version is the most appropriate that is descriptive for the majority of synthesis development processes. Omitting certain steps can potentially expedite the development process, but bear the risk of failure through programming errors or unforeseen challenges. This protocol is designed for both new and experienced users of automated radiosynthesizers. We feel that mock runs (“cold runs”) are always a good idea from a safety and efficiency point of view. Optimization runs are often needed when translating a literature protocol (either manual or on a different automated synthesizer) as typically the

reaction conditions require slight modifications (temperatures, times, etc.) for the best performance. The validation runs are a required procedure (stipulated in regulatory requirements) if one is producing tracers for clinical use. We have revised the discussion part to better reflect and explain the critical steps within the protocol.

SUPPLEMENTARY MATERIAL

Automating a positron-emission tomography (PET) radiotracer synthesis protocol for clinical production

Eric Schopf[1,†], Christopher M. Waldmann[2,3,†], Jeffrey Collins[2,4], Christopher Drake[1], Roger Slavik[2,3,*], R. Michael van Dam[2,4,*].

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[*]Corresponding authors

Contents

1 Manual synthesis of [^{18}F]CFA

The manual radiosynthesis of [^{18}F]CFA was originally described by Shu *et al.* [25] as a procedure using two reaction vessels, intermediate silica cartridge purification and a final HPLC purification step. In brief, [^{18}F]fluoride was first separated from [^{18}O]H_2O by trapping the [^{18}F]fluoride on a quaternary methylammonium (QMA) cartridge and then eluting with a solution of K_2CO_3 and Kryptofix $K_{2.2.2}$ (K222) into the first reaction vessel. Three steps of azeotropic drying with acetonitrile (MeCN) yielded the anhydrous [^{18}F]KF/K222 complex. Precursor (6 mg in 0.6 mL MeCN) was added to the vessel and reacted at 110° for 25 min. The mixture was then passed through a silica cartridge and eluted in 4 steps (2 mL each) into the second reaction vessel. After evaporation, 0.5 mL MeCN was added prior to addition of 1M HCl (1 mL). After the deprotection reaction at 100 °C for 5 min, the reaction mixture was diluted and purified on a semi-preparative HPLC system to yield the final product.

2 Details of automated synthesis program

The programs (unit operations and parameter values for each) for the automated synthesis and formulation of [^{18}F]CFA are summarized in **Tables S1** and **S2**, respectively. The preparation of reagents for the automated synthesis of [^{18}F]CFA is summarized in **Table 1** of the main paper.

Step	Operation	Source	Destination	Duration (s)	Temp. (°C)	Pressure (psi)	Other Parameters
1	Trap Isotope	Source vial	Reactor 1	120	--	7	
2	Elute Isotope	Reagent 1	Reactor 1	90	--	7	
3	Evaporate	Reactor 1	--	150	110	10	• Stirring (210s at 500rpm after 0s delay) • Cooling: 35C
4	Elute Isotope	Reagent 2	Reactor 1	60	--	7	
5	Evaporate	Reactor 1	--	150	110	10	• Stirring (95s at 500rpm after 0s delay) • Cooling: 35C
6	Add Reagent	Reagent 3	Reactor 1	15	--	3	• Add needle: 1 • No stirring
7	Evaporate	Reactor 1	--	300	110	10	• Stirring (300s at 500rpm after 0s delay) • Cooling: 40C
8	Add Reagent	Reagent 4	Reactor 1	15	--	5	• Add needle: 1 • No stirring
9	React	Reactor 1	--	600	120	--	• Seal position: 1 • Stirring (600s at 500rpm after 0s delay) • Cooling: 35C
10	Transfer	Reactor 1	Reactor 2	60	--	10	• Flow path: elute • Stirring (500rpm at source, 500rpm at destination)
11	Add Reagent	Reagent 5	Reactor 1	15	--	3	• Add needle: 2 • No stirring
12	Transfer	Reactor 1	Reactor 2	60	--	10	• Flow path: elute • Stirring (500rpm at source, 500rpm at destination)
13	Evaporate	Reactor 2	--	120	100	10	• Stirring (120s at 500rpm after 0s delay) • Cooling: 35C
14	Add Reagent	Reagent 6	Reactor 1	15	--	3	• Add needle: 2 • Stirring (10s at 500rpm after 15s delay)
15	Transfer	Reactor 1	Reactor 2	60	--	10	• Flow path: elute • Stirring (500rpm at source, 500rpm at destination)
16	Evaporate	Reactor 2	--	120	100	10	• Stirring (120s at 500rpm after 0s delay) • Cooling: 35C
17	Add Reagent	Reagent 7	Reactor 1	15	--	3	• Add needle: 2 • No stirring
18	Transfer	Reactor 1	Reactor 2	60	--	10	• Flow path: elute • Stirring (500rpm at source, 500rpm at destination)
19	Evaporate	Reactor 2	--	270	100	10	• Stirring (270s at 500rpm after 0s delay) • Cooling: 40C

20	Add Reagent	Reagent 8	Reactor 2	15	--	5	• Add needle: 1 • Stirring (25s at 500rpm after 15s delay)
21	Add Reagent	Reagent 9	Reactor 2	15	--	5	• Add needle: 1 • No stirring
22	React	Reactor 2	--	300	100	--	• Seal position: 2 • Stirring (300s at 500rpm after 0s delay) • Cooling: 40C
23	Add Reagent	Reagent 10	Reactor 2	15	--	5	• Add needle: 1 • No stirring
24	Transfer	Reactor 2	HPLC P/F Module: Valve 1	0	--	3.5	• Loop loading mode: manual • Flow path: PF module • Stirring (500rpm at source)
25	Purification	--	Product collect	--	--	--	• Pump mode: isocratic • Solvent: 85% A, 15%B • Flow rate: 5mL/min • UV detection wavelength: 263 nm

Table S1. Detailed sequence of unit operations to perform the automated synthesis and purification of [^{18}F]CFA on the ELIXYS FLEX/CHEM radiosynthesizer and PURE/FORM module. The details of each reagent are described in the main text (**Figure 4**). For the "Purification" unit operation, the ELIXYS PURE/FORM system is equipped with a semi-preparative C-18 column (Gemini, 5 µm, 250 mm x 10 mm, Phenomenex, Torrance, CA, USA), Mobile Phase A is 25 mM ammonium acetate and Mobile Phase B is EtOH.

Step	Operation	Duration(s)	Pressure (psi)	Volume (mL)	Other Parameters
1(a)	Formulation	0	0	-	Mode: Trap
1(b)	Formulation	--	--	--	Mode: Rinse
1(c)	Formulation	--	--	X	Mode: Elute
1(d)	Formulation	--	--	Y	Mode: Reconstitute

Table S2. Detailed sequence of unit operations to perform the automated formulation of [^{18}F]CFA with the ELIXYS PURE/FORM module. In this sequence, the first two sub-operations, Trap and Rinse, are skipped by specifying duration of 0 s and volume of 0 mL, respectively. The Elute and Reconstitute sub-operations must be adjusted to dispense the correct volumes based on the total collected volume of the product after HPLC purification. Volume of HPLC product collected = (5 mL/min) x (1/60) x (Fraction collection duration in s). The Elute operation is configured to dispense a volume of X mL of concentrated sodium chloride (90 mg/mL), where X = (0.096) x (Volume of HPLC product collected). The Reconstitute operation is configured to dispense a volume of Y mL of 0.9% saline solution, where Y = (0.5) x (Volume of HPLC product collected).

3 Setting parameter values for unit operations

The following figures illustrate the programmable parameters for each type of unit operation.

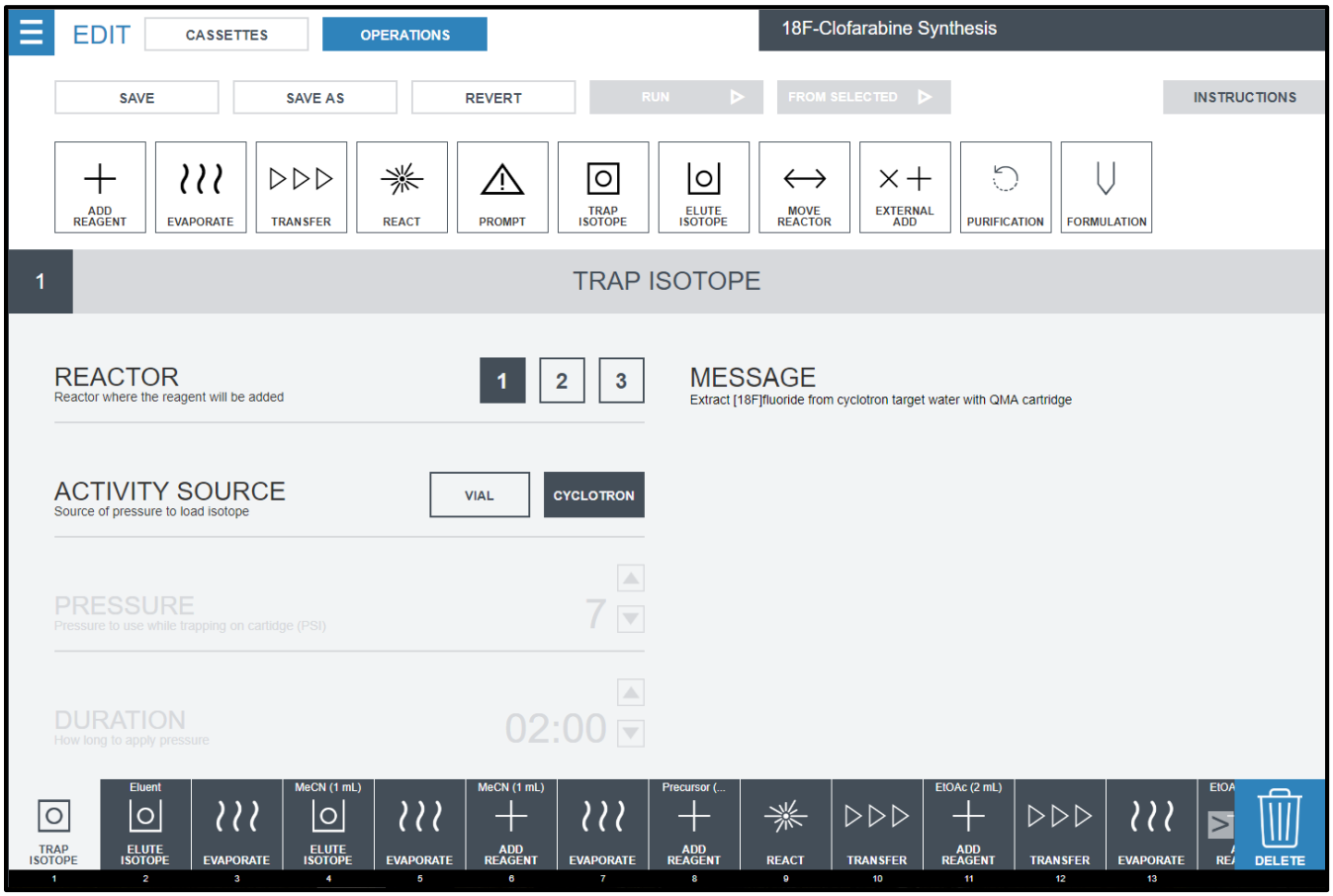

Figure S1. Detail of the Trap Isotope unit operation programming. This unit operation will trap activity, from a source vial or directly from a cyclotron, onto a preconditioned QMA cartridge connected to the cassette and collect the $[^{18}O]H_2O$ in a sealed container. If the activity is initially in a source vial, the FLEX/CHEM will deliver nitrogen at the programmed pressure for the selected duration, through a special gas delivery line, to push the activity onto the QMA cartridge.

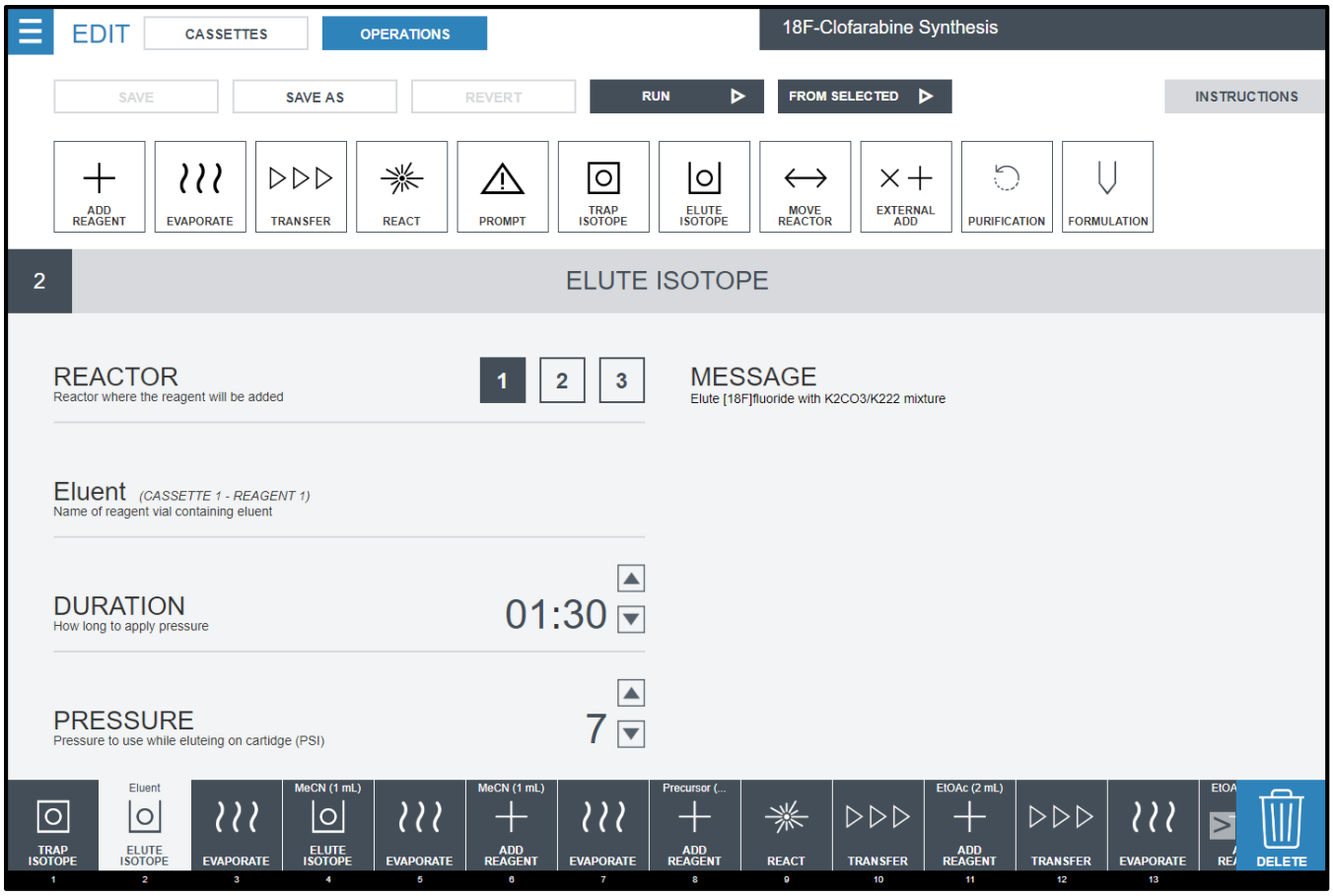

Figure S2. Detail of the Elute Isotope unit operation programming. The operator selects which reagent to use as the eluent solution and specifies the reactor to which the eluate will be directed. The duration and pressure of the operation are programmed as well.

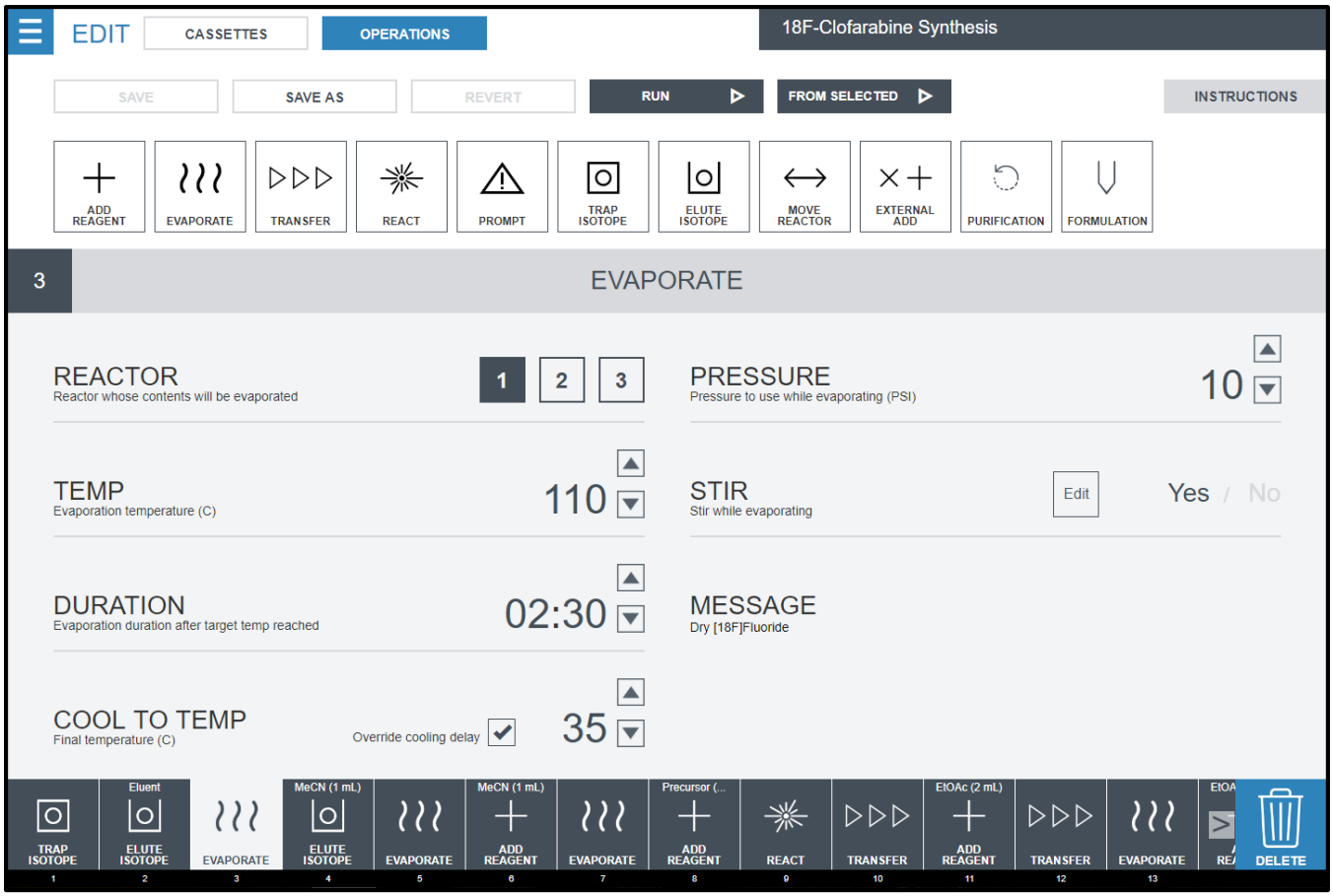

Figure S3. Detail of the Evaporate unit operation programming. The operator selects the desired reactor, evaporation temperature, duration, and pressure of the "sweep gas". The operator can select to have the reactor stirring during the operation. Finally, when the evaporation is finished, the FLEX/CHEM will cool the reactor to the programmed "Cool to Temp" temperature. By default the cooling is extended if the programmed temperature is greater than 70°C by [0.91*T – 43.6] min, where T is the programmed temperature in °C. This accounts for the delay between cooling of the reactor body (measured by temperature sensor) and liquid in reaction vessel (not measured). If "Override cooling delay" is selected, no additional time will be added.

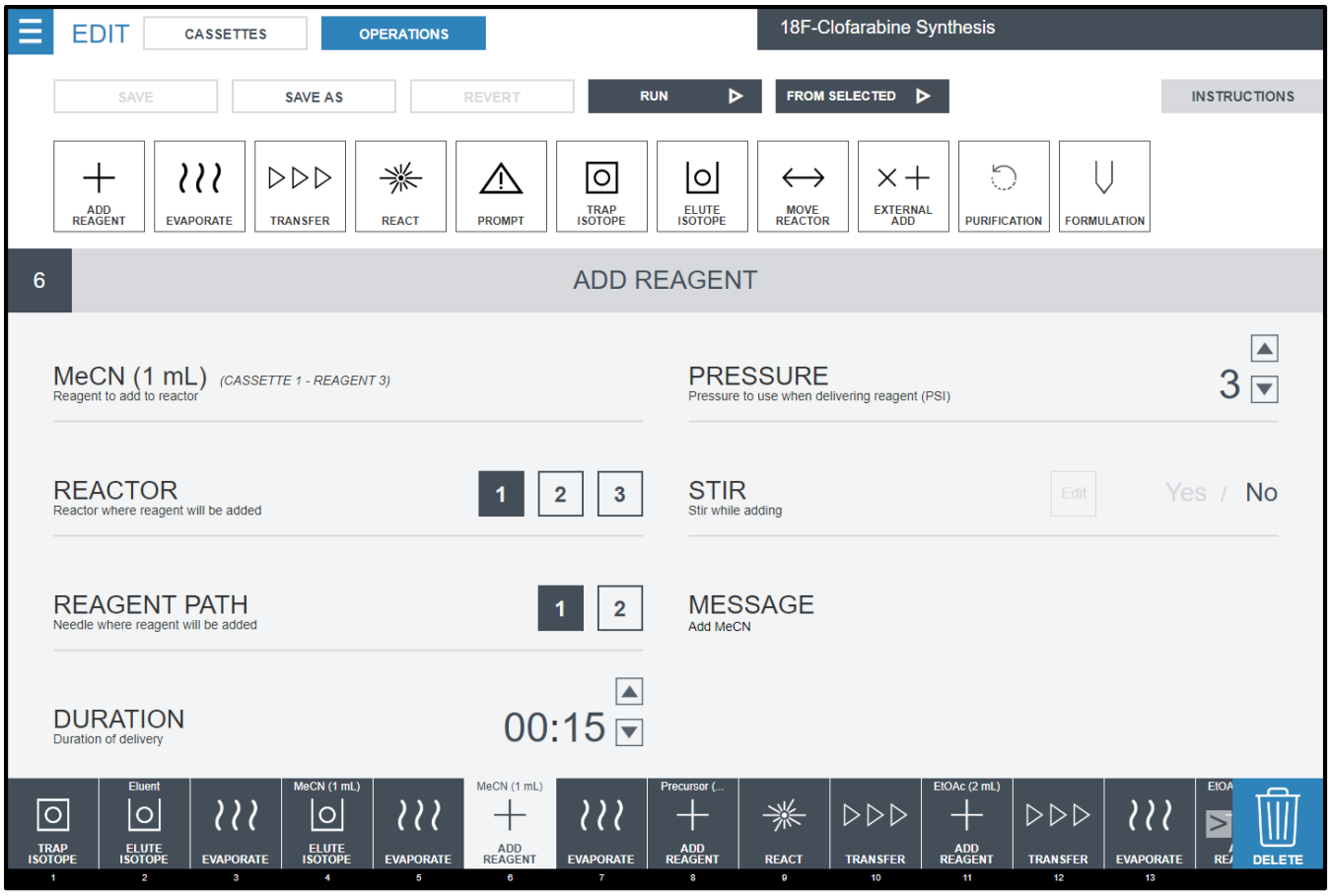

Figure S4. Detail of the Add Reagent unit operation programming. The operator selects which reagent from the "Cassettes" tab (**Figure 4** in main text) is added into the chosen reactor via the selected reagent pathway. The duration and pressure are programmed as well. The operator can choose to stir the vial during or after addition, or to skip stirring.

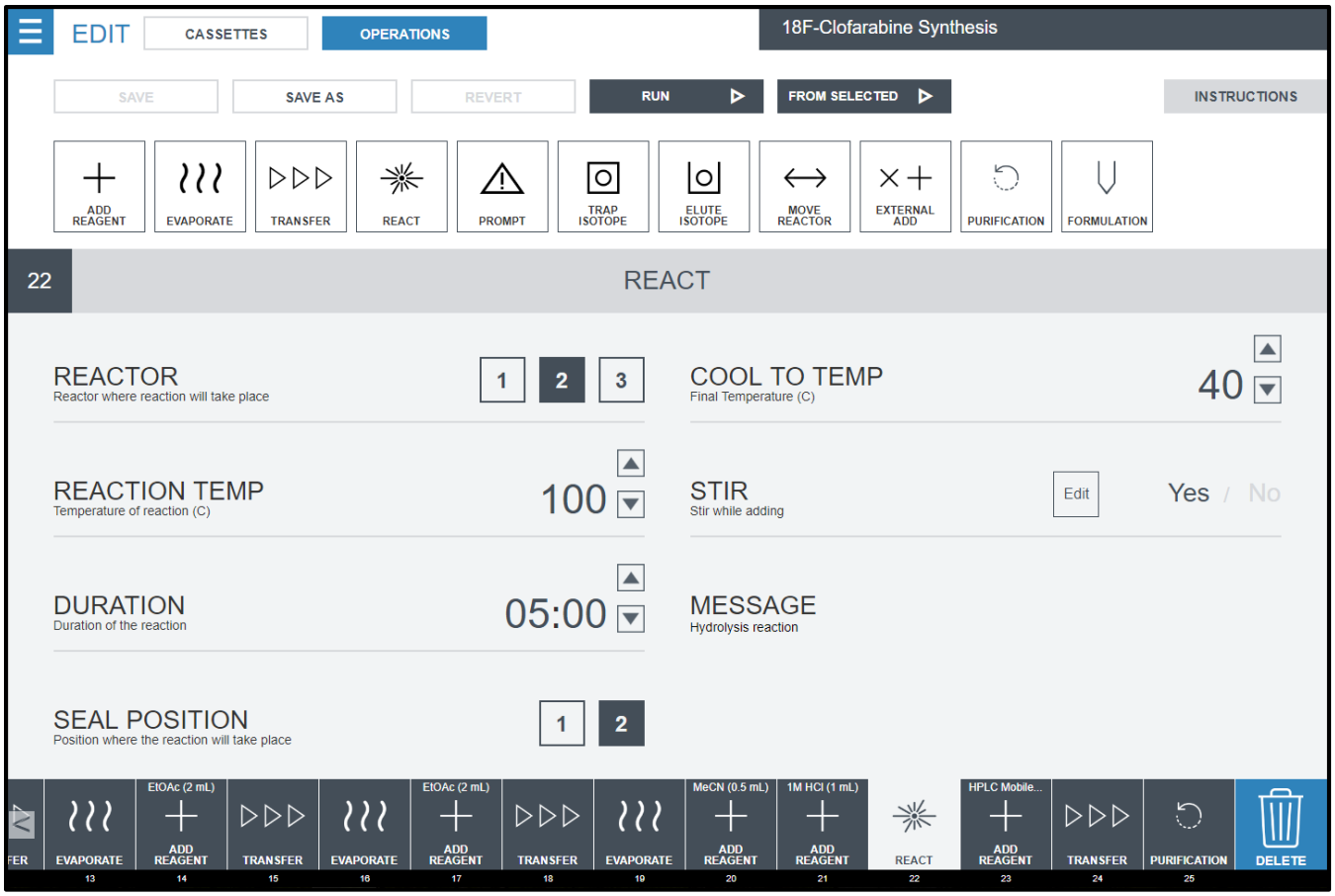

Figure S5. Detail of the React unit operation programming. This unit operation moves the selected reactor to one of two (selectable) seal position on the cassette. The reaction vessel will then be heated to the programmed reaction temperature for the desired duration. The operator programs a desired temperature to which to cool the reactor vial contents after the reaction is complete. A temperature delay will be applied to the cooling time as described for the Evaporate unit operation (**Figure S3**). Finally, the operator can select whether or not to stir the reactor vial during the operation.

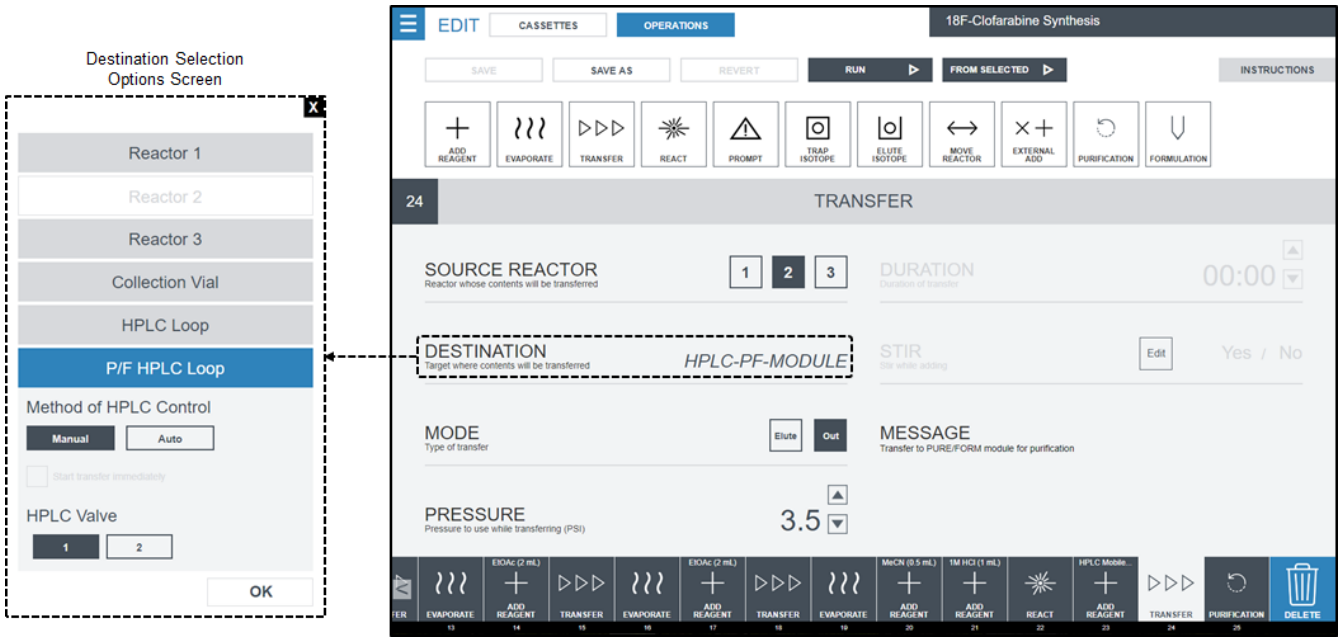

Figure S6. Detail of the Transfer unit operation programming. This unit operation will move the reaction vessel under the dip tube and raise the vial such that the top of the vessel is sealed and the dip tube touches the bottom of the vessel. The contents of the reactor vial will be routed through the cassette fluid pathway chosen to the selected destination, driven by the programmed gas pressure for the programmed duration. In this example, the destination is the purification/formulation module (i.e. injection loop). Because the transfer destination is an HPLC injection loop, the duration is not defined here. Instead, in "Manual" mode, the operator selects when to inject the sample after manually loading the injection loop. "Auto" mode uses the built-in liquid sensors to determine when the entire sample has been loaded into the injection loop and will automatically switch the injection valve to the inject position. If the transfer destination was a reactor, the operator could select the duration and whether or not to stir the reactor vial after the contents have been transferred. In this case, one would also specify whether the transfer is performed in "trap" or "elute" mode (i.e., if the reactor contents are passed through a solid-phase extraction cartridge during the transfer). If no cartridge is used, "elute" would be selected.

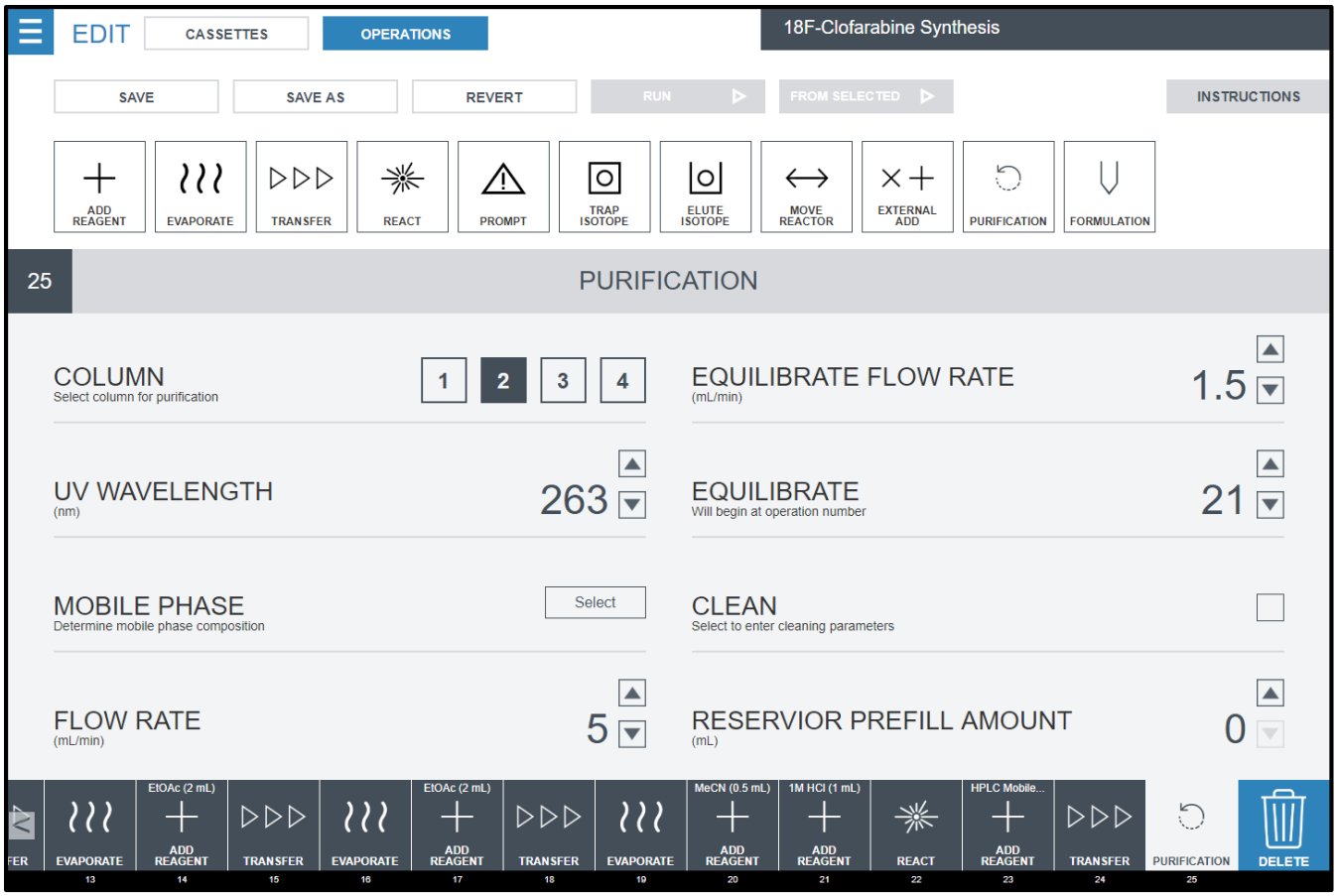

Figure S7. Detail of the Purification unit operation programming. This unit operation controls the HPLC sub-system of the purification/formulation module. First, a column position is selected from the 4 available columns on the column selector valve. The UV detector wavelength is then selected, between 200 and 600 nm. The “Mobile Phase” select button allows the operator to create a solvent program for the quaternary gradient pump to follow; this may be an isocratic or gradient program. The flow rate during the unit operation is then programmed. The module has an equilibration feature where the UV detector lamp will be powered on and the HPLC pump will equilibrate the HPLC sub-system at the desired point in the program. (The flow rate and the unit operation number where the equilibration will begin can be defined.) The “Clean” checkbox will run an automated cleaning of the HPLC sub-system if checked. Finally, the “Reservoir Prefill Amount” allows the operator to specify a volume of diluent that is preloaded in the dilution reservoir to enable calculation of the total volume it contains; this feature is used to alert the operator if the dilution reservoir is at risk of overflowing during the product collection. Since the dilution reservoir and subsequent solid-phase extraction components are not used during the [^{18}F]CFA synthesis, this value is set to 0.

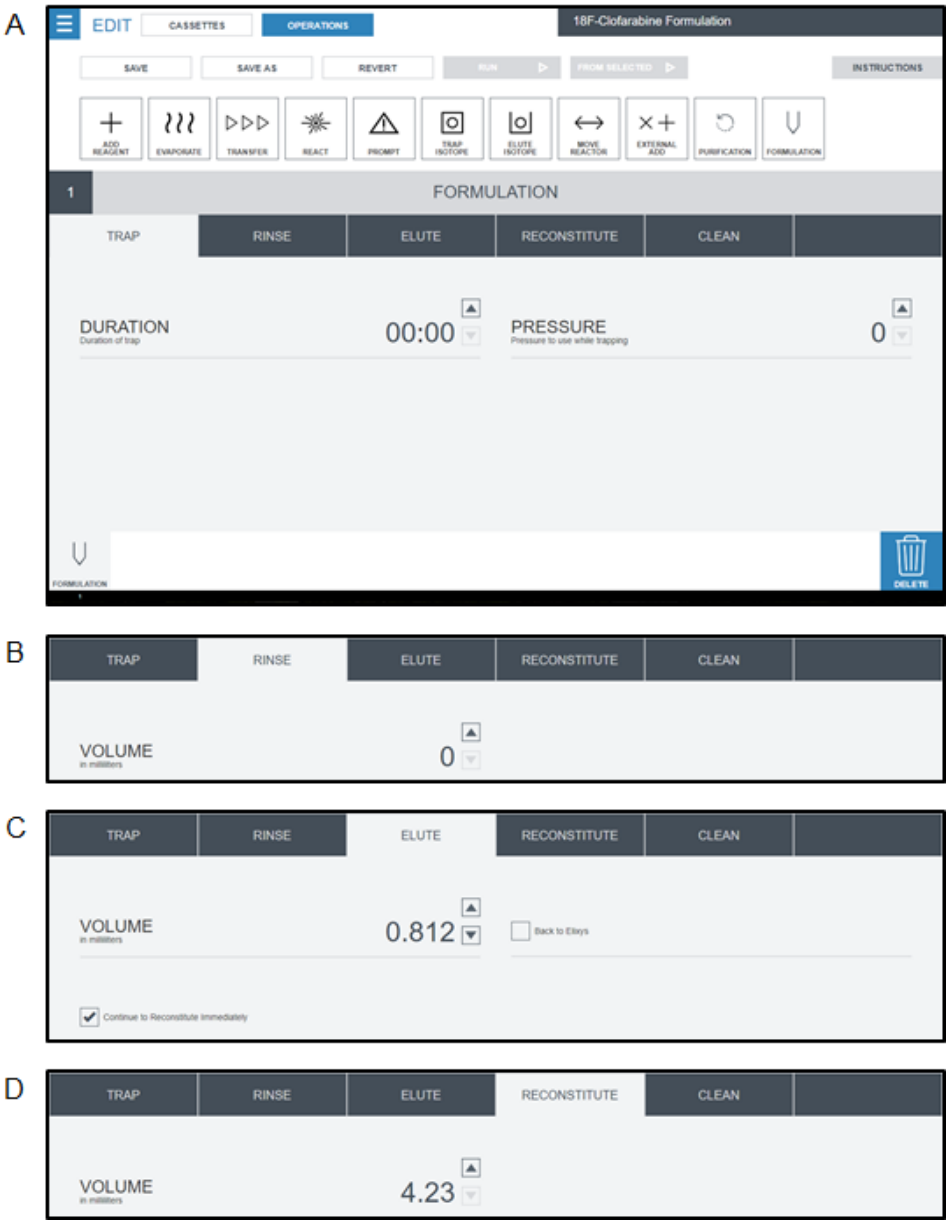

Figure S8. Detail of the Formulation unit operation programming. The Formulation unit operation consists of four sub-operations designed to perform solvent exchange from the HPLC mobile phase to an injectable solution via solid-phase extraction (SPE). (A) The Trap sub-operation pressurizes the dilution reservoir, forcing the diluted product out of the reservoir and through an SPE cartridge where it is trapped; the solvent is routed to waste. (B) The Rinse sub-operation rinses the SPE cartridge containing the trapped product, typically with water, to remove all traces of HPLC mobile phase. (C) The Elute sub-operation is used to elute the trapped product off of the SPE cartridge and into a final product vial. (D) The Reconstitute sub-operation is used to dilute the reformulated product to reduce EtOH content. In the synthesis of [^{18}F]CFA, formulation can be performed via dilution and no SPE cartridge is needed. The Trap and Rinse sub-operations are thus not used, but the Elute operation is used to supply concentrated NaCl (90 mg/mL) to adjust isotonicity, and the Reconstitute operation is used to supply saline (0.9%) to reduce EtOH content to acceptable levels. In the example shown (validation run #2), the collected product volume of 8.46 mL requires 0.812 mL of concentrated NaCl (90 mg/mL), and 4.23 mL of saline (0.9%). Calculations are performed using the NaCl equivalent method.

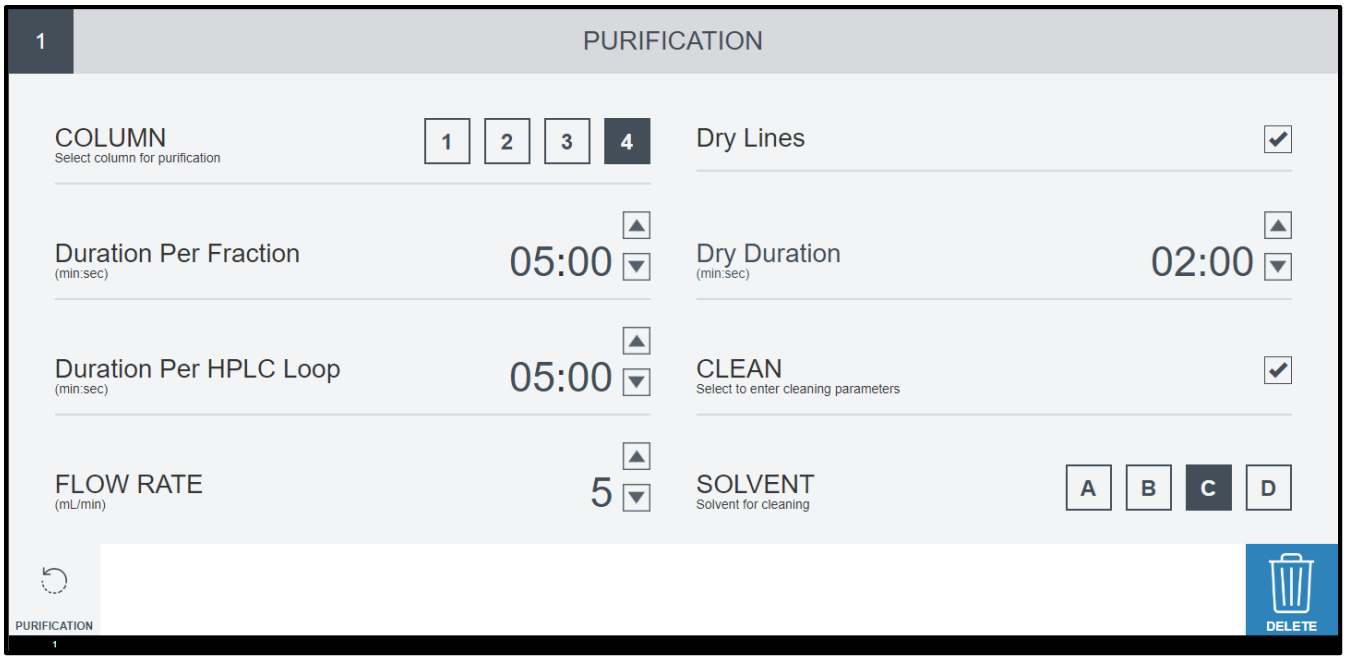

Figure S9. Unit operation configuration of an automated cleaning process for the Purification sub-system (using the Purification unit operation). Mobile phase (input "C" selected here) will be pushed through the system through the selected column (position 4) at the entered flow rate (5 mL/min). The system will switch between the two sample loops after a specified time (5 min). Afterwards, the selection valve will be actuated through each of its outputs, holding for a specified duration at each output (5 min). Finally, if desired ("Dry Lines" checkbox), the system will use nitrogen to dry the output lines from the selection valves, drying each line for a specified duration (2 min).

Figure S10. Unit operation configuration of an automated cleaning process for the Formulation sub-system (using the Formulation unit operation). The sub-system cleaning uses pre-set parameters to rinse all input/output lines and fluid paths with ethanol and dry them.

References

1. Shu, C.J. *et al.* Novel PET probes specific for deoxycytidine kinase. *Journal of Nuclear Medicine*. **51** (7), 1092–1098, doi: 10.2967/jnumed.109.073361 (2010).