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TITLE:
Automated Synthesis and Uptake Analysis of D-[Methyl-^{11}C]-Methionine

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SUMMARY:
Here, we present a protocol for the synthesis of D-[methyl-^{11}C]-methionine, a metabolic positron emission tomography tracer for bacteria, and in vitro evaluation in *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. The synthesis involves a single synthetic step on an automated module, followed by microcentrifuge filtration.

ABSTRACT:
Positron emission tomography (PET) has emerged as a vital molecular imaging modality that provides metabolic insights that are essential for the diagnosis and treatment of disease. Among the many useful applications of PET, imaging of infection has gained much traction in the last decade. To this end, we describe the synthesis and in vitro evaluation of D-[methyl-^{11}C]-

methionine, a potent metabolic tracer for living bacteria. D-[methyl-^{11}C]-methionine was synthesized from [^{11}C]methyl iodide ([^{11}C]CH_3I) using an in-loop synthesis method in an automated synthesis module. The radiotracer was analyzed by chiral high-performance liquid chromatography (HPLC) to determine its radiochemical identity and purity and then subjected to in vitro analysis using a rapid method to determine uptake in bacteria cells. The workflow described demonstrates the importance of communication and time management when developing new radiotracers, especially with short half-life isotopes such as carbon-11 (^{11}C) ($t_{1/2}$ = 20.4 min).

INTRODUCTION:

The use of new imaging techniques to detect infection is an emerging field and in recent years, PET imaging has been a leading innovator with several tracers specifically sensing living bacteria[1-8]. D-[methyl-^{11}C]-methionine is a first-generation, metabolic PET tracer that images live bacterial infection by targeting peptidoglycan, a key cell wall component in both gram-positive and gram-negative bacteria pathogens[9]. Carbon-11 labelling of D-homocysteine easily affords the PET tracer in a single step from [^{11}C]CH_3I (**Figure 1**). [^{11}C]CH_3I is synthesized from [^{11}C]-carbon dioxide ([^{11}C]CO_2), which is cyclotron-generated. The synthetic process involves [^{11}C]CO_2 being reduced to [^{11}C]methane ([^{11}C]CH_4), which is subsequently reacted with iodine to yield [^{11}C]CH_3I.

The above process takes approximately 11 min, which is important because ^{11}C only has a half-life of 20.4 min. In general, for PET studies to yield enough radiotracer for in vitro studies, the radiosynthesis must not take any longer than 2–3 half-lives (40–60 min for ^{11}C) of the radioisotope[10]. As D-[methyl-^{11}C]-methionine is actively metabolized, high bacterial accumulation is anticipated due to enzymatic turnover compared to receptor-binding tracers. One major drawback of the use of the cyclic precursor (D-homocysteine thiolactone-HCl) is base-catalyzed, undesired epimerization resulting in the formation of the L-enantiomer of [methyl-^{11}C]-methionine.

Much like radiosynthesis, rapid pharmacokinetics is essential as it is important that localization of the tracer to regions of interest (cells, tissues) be observed quickly. For ^{11}C, imaging beyond 1–2 h is not feasible due to signal loss from decay. Therefore, quick internalization and retention is required for metabolic tracers like D-[methyl-^{11}C]-methionine. Any additional experiments will also require rapid processing for in vitro data collection to help identify tracers that will be successful in vivo.

Here, we describe the automated in-loop synthesis of D-[methyl-^{11}C]-methionine using ^{11}C methylation and the rapid analytical technique used for in vitro assessment of bacterial accumulation. The goal of this work is to provide an overview of the efficient methods needed to synthesize and analyze a ^{11}C radiotracer for PET molecular imaging studies. First, we review a plan to ensure the expedited chain of custody for the radioactivity as it navigates the synthesis, qualitative analysis, and in vitro experiments. Second, we describe a stepwise walkthrough of the preparation of the radiotracer and subsequent quality control (QC). Third, a workflow is provided for a rapid in vitro assay for the determination of bacterial uptake.

PROTOCOL:

CAUTION: In the following protocol, there are multiple manipulations that require the handling of radioactivity. It is extremely important that every interaction with radioactivity be executed in agreement with the Radiation Safety Department of the institute and the respective national guidelines. It is mandatory to minimize the exposure to ionizing radiation for the operators involved following the "as low as reasonably achievable" (ALARA) principle.

NOTE: "Current Good Manufacturing Procedures" (cGMP) are written into this protocol given that the synthesis and QC are done in the Clinical Production lab. cGMP is not required for preclinical use of this product or any other product. Requirements differ from facility to facility and will be referenced when they are not needed. Each synthesizer has module-specific programs to generate the [^{11}C]methyl iodide as well as product-specific programs for tracers. Herein, the "DMet Loop" program was modified using the program provided by the vendor and will need to be developed according to the facility's capabilities.

1. Time management and planning of the experiment

NOTE: The ^{11}C nuclide has a short half-life of 20.4 min, making time management extremely important for all subsequent experiments to minimize loss of radioactivity (**Figure 2**). Ensure that specific personnel are responsible for different aspects of each experimental procedure, and coordinate between them with respect to timing and execution. For this experiment, four personnel are necessary: one for synthesis, one for QC, and two for preparation of the radionuclide and bacteria for the uptake assay and for performing the assay. To plan the experiment:

1.1. Organize time for the synthesis of the radiopharmaceutical based on the availability of isotope production and the synthesizer.

1.2. Inform the QC operator of the release criteria and specifications for preclinical use.

1.3. Ensure all components of the uptake assay are prepared.

1.4. Ensure a well-established chain of custody for the radiopharmaceutical from synthesis to experimental completion.

2. Automated synthesis of D-[methyl-^{11}C]-methionine for preclinical use

NOTE: Prepare the final product vial (FPV)-cGMP requirement at this facility.

2.1. Clean the laminar flow hood in accordance with cGMP standards, using standard operating procedures within the radiopharmaceutical facility.

2.2. Label air settling and contact plates for monitoring sterility.

2.3. Spray all components with sterile isopropyl alcohol (IPA), and place in the laminar flow hood.

2.4. Prepare a 20 mL evacuated vial with a sterile alcohol pad, vent needle, and 0.22 µm filter connected to an 18 G needle.

2.5. Perform environmental monitoring.

2.6. Dissolve 0.2 g of sodium dihydrogen phosphate in 1 mL of water to make a 0.2 g/mL solution. Add 1 mL of ethyl alcohol (EtOH) to a vial, then add 1 mL of 1 M NaOH to that vial, and label the vial as "50/50 0.5 M NaOH in water/EtOH".

2.7. Synthesis module preparations

2.7.1. Clean the synthesis module in accordance with the standard operating procedure (SOP) using ultra-high purity (UHP) water, sterile water for injection (SWFI), acetone, and EtOH; empty the vacuum trap, and clean the 2 mL Teflon loop with 5 mL water, 2 × 5 mL acetone, followed by drying with nitrogen gas flow of 30 mL/ min for 10 min. Verify that the hydrogen and helium gas tanks are open.

2.7.2. Perform a manual leak check of the system prior to synthesis (**Figure 3**).

2.7.2.1. Replace the large round bottom flask with a 10 mL dilution reservoir and small stir bar. Adjust tube lengths accordingly to ensure that the tube reaches the bottom of the 10 mL vial and that the vial is standing vertically.

2.7.2.2. Remove the outlet of V7, and attach it to V14 in the dilution vessel.

2.7.2.3. Initiate the manual system software, and leak check the system with the 2 mL loop placed between the reactor and V8 (**Figure 3**).

2.7.3. Perform a final product line sanitization-cGMP if delivering to a dispensing cell.

2.7.3.1. Attach the outlet of V13 to the final product delivery line in the dispensing cell.

2.7.3.2. Place 5 mL of SWFI in reservoirs 5 and 6.

2.7.3.3. Add SWFI into the product intermediate vial through manual manipulations of the software.

2.7.3.4. Transfer the contents of the product intermediate vial through the delivery line into the dispensing cell and to the waste vial. Confirm the contents are in fact transferred to the dispensing cell and into the waste vial.

2.7.3.5. Add 5 mL of EtOH to reservoirs 5 and 6. Repeat transfers in steps 2.7.3.3 and 2.7.3.4 through the product intermediate vial and to the dispensing cell.

2.7.3.6. Add 10 mL of SWFI in reservoir 5 and repeat the transfers in steps 2.7.3.3 and 2.7.3.4. After the lines are clear of liquid, continue to purge with gas for 5 min to dry the delivery line.

2.7.3.7. Close all valves on the synthesizer, exit the manual mode, and reset the system.

2.7.4. Fill the cooling dewar with liquid nitrogen, and tighten the fittings.

2.7.5. Add liquid nitrogen to the vacuum trap dewar (previously emptied during the cleaning of the module).

2.7.6. Place 3 mL of saline in reservoir 2.

2.7.7. Place 3 mL of saline in reservoir 3.

2.7.8. Place 2 mL of saline in the product intermediate vial.

2.7.9. Place 300 µL of 0.2 g/mL NaH_2PO_4 solution in the product intermediate vial.

2.7.10. Activate a solid phase extraction (SPE) C18 light cartridge with 5 mL of EtOH, then 10 mL SWFI, and place in the SPE position (**Figure 3**).

2.7.11. Ensure V7 to the right of V14 is attached such that V2 and V3 rinses pass into the dilution vial.

2.7.12. Ensure that the round bottom flask has been replaced with a 10 mL dilution reservoir.

2.7.13. Attach V12 to the overflow vial to collect the waste from loading the SPE cartridge.

2.7.14. Attach the final product line to the dispensing cell delivery line.

2.7.15. Ensure that the MeI trap is inserted into the heater (**Figure 3**).

2.8. Precursor preparation

2.8.1. Remove the precursor vial from the refrigerator and allow it to reach room temperature (at least 20 min before dissolution).

2.8.2. Dissolve the precursor in “50/50 0.5 M NaOH in water/EtOH” such that the final concentration is 1.25 mg in 100 µL.

2.8.3. Using a 1 mL syringe with 25 G × 5/8" needle, withdraw 100 µL from the vial, and slowly add to the 2 mL Teflon loop, followed by 200 µL of air.

2.8.4. Adjust the loop between the reaction vessel and V8 such that the side where the precursor was added from is toward V8 to allow the solution to spread into the loop before reaching the reactor when [^{11}C]methyl iodide is sent to the reactor.

2.9. Synthesis

2.9.1. Start the "DMet Loop" program.

2.9.1.1. Exit the manual mode and reset the software.

2.9.1.2. Enter batch number (yymmddDMETx), where x is the sequential batch number of the day.

2.9.1.3. Depending on the desired activity, start the program such that trap conditioning is done 20–30 min before the end of bombardment (EOB).

2.9.1.4. Confirm that the actions in the following preparation list are performed.
V1: Empty
V2: 3 mL of saline are added.
V3: 3 mL of saline are added.
NaH_2PO_4 solution (0.2 g/mL, 300 µL) is added into the product intermediate vial
Saline (2 mL) is added into the product intermediate vial.
SPE C18 cartridge is conditioned and placed in the module.
Precursor, as prepared above, is loaded onto the loop and attached between V8 and the reaction vessel.

2.10. Cyclotron-produced [^{11}C]CO_2

2.10.1. Ensure that the magnet is turned on, and that the vacuum is appropriate. Empty and refill the carbon target.

2.10.2. Choose the appropriate target and start the production with a current of 50–55 µA. When ready, select **Start Irradiation**, and wait for beam-on. Note that this production needs to be done concurrently with the presynthesis (module) preparation as the bombardment takes 20–40 min.

2.10.3. When the desired activity is reached, and the synthesizer is ready to receive activity, select **Delivery**, and document the EOB.

2.10.4. MeI production

2.10.4.1. Condition the nickel oven until the beam is on target.

2.10.4.2. Start cooling the methane trap 5 min before receiving $[^{11}C]CO_2$.

2.10.4.3. Document the EOB time when starting the transfer of activity.

2.10.4.4. Continue after the $[^{11}C]CO_2$ transfer is complete.

2.10.4.5. Continue the synthesis continues according to the time list.

NOTE: Following the delivery of $[^{11}C]CO_2$ from the cyclotron, the $[^{11}C]CO_2$ from the cyclotron is trapped on a column of molecular sieves and nickel at room temperature. The column is flushed with H_2 gas, sealed to pressurize, and finally heated to 350 °C to reduce $[^{11}C]CO_2$ to $[^{11}C]CH_4$. $[^{11}C]CH_4$ is then transferred into a methane trap that is previously cooled to -80 °C with liquid nitrogen. The methane trap is warmed to release $[^{11}C]CH_4$, which enters a recirculation loop containing an iodine reservoir at 90 °C at the entry point of the MeI oven set to 750 °C. The $[^{11}C]CH_4$ is converted to $[^{11}C]CH_3I$ over a period of 5 min, and the converted $[^{11}C]CH_3I$ is removed from the recirculation loop by a column that traps $[^{11}C]CH_3I$ at room temperature, but allows $[^{11}C]CH_4$ to pass through. The $[^{11}C]CH_3I$ is released upon heating of the column to 180 °C. The $[^{11}C]CH_3I$ is flowed through the 2 mL Teflon loop, previously coated with the precursor for 70–90 s at a rate of 15 mL/min. The system is then closed to react for 1 min at room temperature. The loop is rinsed with 2 additions of 3 mL of saline into the 10 mL dilution flask, which is passed through the SPE C18 cartridge into the intermediate product vial previously charged with 300 µL of 0.2 g/mL NaH_2PO_4 and 2 mL of saline. This is transferred over a sterilizing filter to the final product vial.

2.10.4.5.1. Note the activity on the detectors or readouts from the cyclotron: $[^{11}C]CO_2$ from the cyclotron, MeI counts, shine in the intermediate product vial, and final product activity.

2.10.5. Post-synthesis

2.10.5.1. Clean up the synthesizer following sufficient decay or on the day after removing the loop and placing all the tubing in the original position.

2.10.5.2. Print rad detector reports and synthesis/ log reports.

2.11. Post-delivery

2.11.1. After the product has been delivered, clear the filter of liquid. Remove the delivery line from the top of the sterilizing filter.

2.11.2. Weigh the final vial and note the weight in the batch record.

2.11.3. Assay the final vial in a dose calibrator. Calculate the concentration (mCi/mL).

2.11.4. Remove the vial from the dose calibrator and take a QC sample (~0.6 mL).

2.11.5. Inoculate sterility tubes (cGMP requirement for human doses, but not done for preclinical studies).

2.11.6. Remove the sterilizing filter and vent needle. Put the final product vial back in the dose calibrator, and start half-life analysis (cGMP requirement, but not required for preclinical studies).

3. Quality control

3.1. Perform a visual inspection: clear, colorless, no particulate matter.

3.2. Perform a filter integrity test (bubble point): ≥50 psi (cGMP studies only).

3.3. Check the radiochemical purity (HPLC), that the radioactive peak corresponds to the standard peak.

3.4. Check the radiochemical purity (HPLC): ≥90%.

3.5. Check the radionuclidic identity (half-life): Run for at least 10 min; $t_{1/2}$ = 19.5–21 min (cGMP studies only).

3.6. Check the radionuclidic purity (MCA): 511 keV peak (cGMP studies only).

3.7. Check the pH: 4.5–7.5.

3.8. Check for bacterial endotoxins: <5 EU/mL (cGMP studies only).

3.9. Check for residual solvents (gas chromatography): EtOH ≤10% (cGMP studies only).

3.10. Start the sterility test: Started within 24 h (cGMP studies only).

4. Uptake assay

NOTE: All handling and experiments containing live bacteria (**Figure 5**) need to be within a designated biosafety cabinet within a biosafety level 2 (BSL-2) laboratory and in accordance with all safe practices and regulations at institutional, state, and federal levels. The majority of pathogenic bacteria are BSL-2.

4.1. Bacterial culture

4.1.1. Remove a single colony of bacteria from an agar plate using a disposable loop and suspend in 50 mL of Luria-Bertani (LB) broth in a 125 mL culture flask.

4.1.2. Clamp the flask in an incubator shaker and agitate at 111 rpm and at 37 °C for 16 h.

4.2. Prepare the bacteria for experiments.

4.2.1. Remove an aliquot of 10 mL, and pellet at 1300 × *g* for 5 min.

4.2.2. Remove the supernatant.

4.2.3. Resuspend the pellet in 10 mL of Ham's F12 medium.

4.2.4. Aliquot the resuspended pellet into 50 mL centrifuge tubes containing a ½ dilution series of "cold" D-methionine ranging from 1 mM to 15.625 µM and 0 added D-methionine. Three experiments (centrifuge tubes) for each concentration are used for a total of 24 experiments.

4.3. Prepare the radiotracer for experiments.

4.3.1. In a syringe, remove 3 mCi of the radiopharmaceutical, and dilute up to 1 mL in Ham's F12 medium in a 2 mL centrifuge tube.

4.3.2. Using a pipet, dispense 33.3 µL of activity to each bacterium-containing 50 mL centrifuge tube.

4.3.3. Cap and seal each 50 mL centrifuge tube.

4.4. Perform the uptake assay.

4.4.1. Place the rack of twenty-four 50 mL centrifuge tubes in an incubator shaker for agitation at 180 rpm for 90 min.

4.4.2. After 90 min, remove 500 µL aliquots from each centrifuge tube, and place into 1.5 mL filtration tubes (for partitioning and analysis) and 1 mL UV/Vis cuvette (for final optical density (OD) determination).

4.4.3. Centrifuge the 1.5 mL filtration tubes at 7,500 × *g* for 5 min.

4.4.4. Wash the collected pellets with 200 µL of phosphate-buffered saline, and centrifuge at 7,500 × *g* for 5 min.

4.4.5. Separate the collected pellets from the filtrates and close all containers.

4.5. Data collection

4.5.1. Analyze the pellets and filtrates individually on a gamma counter. The gamma counter measures a window of 480–558 keV for 30 s for each sample.

4.5.2. Analyze the aliquoted cuvettes on a UV/Vis spectrophotometer set to a wavelength of 600 nm.

4.6. Data analysis and processing

4.6.1. Use the gamma counter to measure the amount of activity in each pellet and filtrate sample.

4.6.2. Use the UV/Vis spectrophotometer to provide the OD of each bacterial sample.

4.6.3. Use the OD to obtain the concentration of colony-forming units (CFU/mL) of the bacteria where ~1 OD_{600} corresponds to 8 $x10^8$ CFU/mL as predetermined from plating and counting of the serial dilution series of each pathogen.

4.6.4. Express the uptake initially as a percentage of activity in the pellet. At time zero, each 500 µL aliquot contains ~4.69 µCi of activity. Multiplication of the percentage provides the µCi of the accumulated radiotracer.

4.6.5. Convert the µCi to Becquerel (Bq) using the conversion factor of 37000 Bq/µCi. The final expression of the cellular uptake data is Bq/million cells obtained from the CFUs and the activity at time zero.

4.6.6. Plot Bq/million cells against the concentration of added "cold" D-methionine (**Figure 6**).

REPRESENTATIVE RESULTS:

The automated in-loop radiosynthesis of D-[methyl-^{11}C]-methionine yielded >99% enantiomeric excess (ee, n = 9), 22% ± 13% decay-corrected radiochemical yield, and >90% radiochemical purity in all cases. The overall synthesis required 20 min to complete, including 15 min dedicated to the synthesis of [^{11}C]methyl iodide. Labeling of the D-homocysteine precursors was completed in only 2–3 min and required passage through a C18 Sep Pak for isolation and purification. A small aliquot (~200 µL) was required for quality control. The remainder was used to carry out subsequent experiments.

Quality control was completed in 30 min. The identity, radiochemical purity, and ee of D-[methyl-^{11}C]-methionine was determined using chiral HPLC with gamma and UV detection (220 nm). A Chirobiotic T2 column (250 mm × 4.6 mm) was used as the stationary phase, and 3:1 acetonitrile/water was used as the mobile phase. All other parameters (filter integrity, endotoxins, sterility, residual solvents) fulfilled the release criteria. The final dose of D-[methyl-^{11}C]-methionine was a clear, colorless solution with a pH of 7. Representative chromatograms are presented in **Figure 4**.

For the in vitro analysis, blocking studies are presented in **Figure 6**. The uptake of D-[methyl-^{11}C]-methionine was measured in three different pathogens: *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* with increasing concentrations of "cold" D-methionine added. As shown, D-[methyl-^{11}C]-methionine was rapidly taken up by all three pathogens with uptake by *S. aureus* and *P. aeruginosa* being 2-fold greater than that by *E. coli*. Uptake into all three pathogens was incrementally decreased with increasing concentrations of the "cold" additive ranging from 15.625 to 1000 µM.

FIGURE AND TABLE LEGENDS:

Figure 1: Synthesis of D-[methyl-^{11}C]-methionine. [^{11}C]-carbon dioxide ([^{11}C]-CO_2), which was cyclotron-generated, was converted to [^{11}C]-methyl iodide ([^{11}C]CH_3I) and then reacted with D-homocysteine to yield D-[methyl-^{11}C]-methionine.

Figure 2: Overview of the workflow. Color-coded workflow illustrating the dedicated task management for the radiosynthesis, quality control, and in vitro experiments for D-[methyl-^{11}C]-methionine.

Figure 3: Radiosynthetic scheme of the automated synthesizer. Modifications to the synthesis module for the in-loop synthesis of D-[methyl-^{11}C]-methionine are as outlined in the above schematic (highlighted in blue).

Figure 4: Representative quality control (QC) of the automated radiosynthesis of D-[methyl-^{11}C]-methionine. The analytical chiral separation illustrating the identity, purity, and enantiomeric composition of the final D-[methyl-^{11}C]-methionine. UV detection is on top and is reported in units of time versus absorption units (AU). Radioactivity detection is in the bottom and is reported in units of time versus counts.

Figure 5: Experimental workflow of high-throughput in vitro analysis. A single bacterial colony was removed from **(A)** a culture plate and added to **(B)** a 50 mL LB culture. The culture was grown for **(C)** 16 h and then reconstituted in **(D)** F12. Subcultures were performed, and **(E)** activity was added and incubated for 90 min. **(F)** Aliquots were taken from each subculture and added to filtration tubes. **(G)** The tubes were centrifuged and washed to pellet the bacteria. **(H)** The filters with the bacteria pellets were removed and capped for separate analysis.

Figure 6: Representative uptake measurements of D-[methyl-^{11}C]-methionine in bacteria. Uptake measurements of D-[methyl-^{11}C]-methionine in three different pathogens: *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* incubated with varying concentrations of "cold" added D-methionine ranging from 15.635 µM to 1000 µM. Uptake was incrementally decreased with increasing concentrations of "cold" D-methionine.

DISCUSSION:

The radiosynthesis of D-[methyl-^{11}C]-methionine was performed in a commercial synthesis module using an in-loop method that yielded superior yields and purities over previously

reported conventional manual approaches (>99% ee, 22% ± 13% decay-corrected radiochemical yield versus 85% ee, 20% ± 1% decay corrected radiochemical yield)[9]. When making radiotracers from either [^{11}C]carbon dioxide or [^{11}C] methyl iodide, is it imperative that a completely closed and inert system be used to prevent "cold" carbon dioxide from the atmosphere participating in the reactions and subsequently lowering the molar activity of the final product. This is more essential for receptor-binding tracers than for metabolic tracers such as D-[methyl-^{11}C]-methionine. To this end, ensuring that all lines are clean and flushed with inert gas is an important step in the preparation of any ^{11}C tracer. As the intermediate steps in the [^{11}C]methyl iodide synthesis are equally susceptible to moisture, preheating all reactors prior to synthesis runs is vital to ensure that the system is completely dry. The synthesis module is equipped with several detectors to monitor the flow and trapping of the [^{11}C]carbon dioxide and [^{11}C]methane during the synthesis.

For in vitro experiments, the most important quality control elements are identity and radiochemical purity, both of which are determined via HPLC. For ^{11}C tracers, it is important to optimize the HPLC conditions to shorten the elution time for the product without compromising the diagnostic capabilities. As shown, the flow rate and solvent composition were optimized to shorten the elution time of D-methionine to <10 min while maintaining separation from L-methionine.

The rapid assay defined here is useful for determining uptake of the radiotracer in free-flowing cells without the need to transfer radioactive liquids between vessels after the aliquot is removed from the experiment. This is a vast improvement for the reproducibility and quality of data as well as for safety. The elimination of unnecessary transfers shortens the duration of the experiment and increases the throughput. Twenty-four filter tubes can be centrifuged at one time, allowing for more data and higher statistical repeats to be accumulated from a single synthesis of D-[methyl-^{11}C]-methionine. Each filter tube produces two components that can be individually analyzed on a gamma counter to measure the amount of activity in the filtrate portion and in the cell pellet. The gamma counter is fully automated and measures each sample in 30 s. Typically, all the pellets are run before the corresponding filtrates. The gamma counter provides decay-corrected values for radioactivity that can be easily exported into spreadsheets for easy processing and graphing. The same work flow can be used to determine the uptake of a tracer in several different pathogens, or for several different incubation periods, or as shown in **Figure 6**, for several different concentrations of "cold" additive. This procedure does possess limitations that require careful consideration. As each sample requires two reads on the gamma counter at 30 s each, 24 samples require 24–30 min to analyze. This already exceeds one half-life of ^{11}C . The time taken for the last sample to be run on the gamma counter is the key to determining the size and scale of the experiment. For the purposes of using ^{11}C , 24 samples are easily manageable.

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DISCLOSURES:

The authors have nothing to disclose.

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8. Parker, M. F. L. et al. Sensing living bacteria in vivo using D-alanine-derived ^{11}C radiotracers. *ACS Central Science.* **6** (2), 155−165 (2020).

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10. Pichler, V. et al. An overview on PET radiochemistry: part 1 - covalent labels - ^{18}F, ^{11}C, and ^{13}N. *Journal of Nuclear Medicine*. **59**, 1350−1354 (2018).

Figure 1

Click here to access/download;Figure;Figure 1.tif

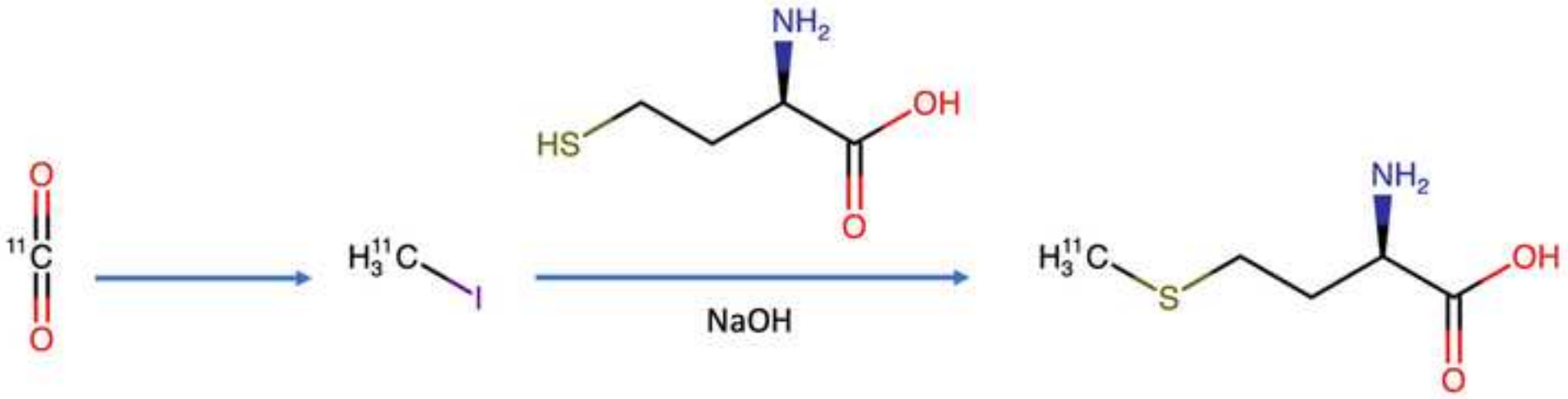


Figure 2

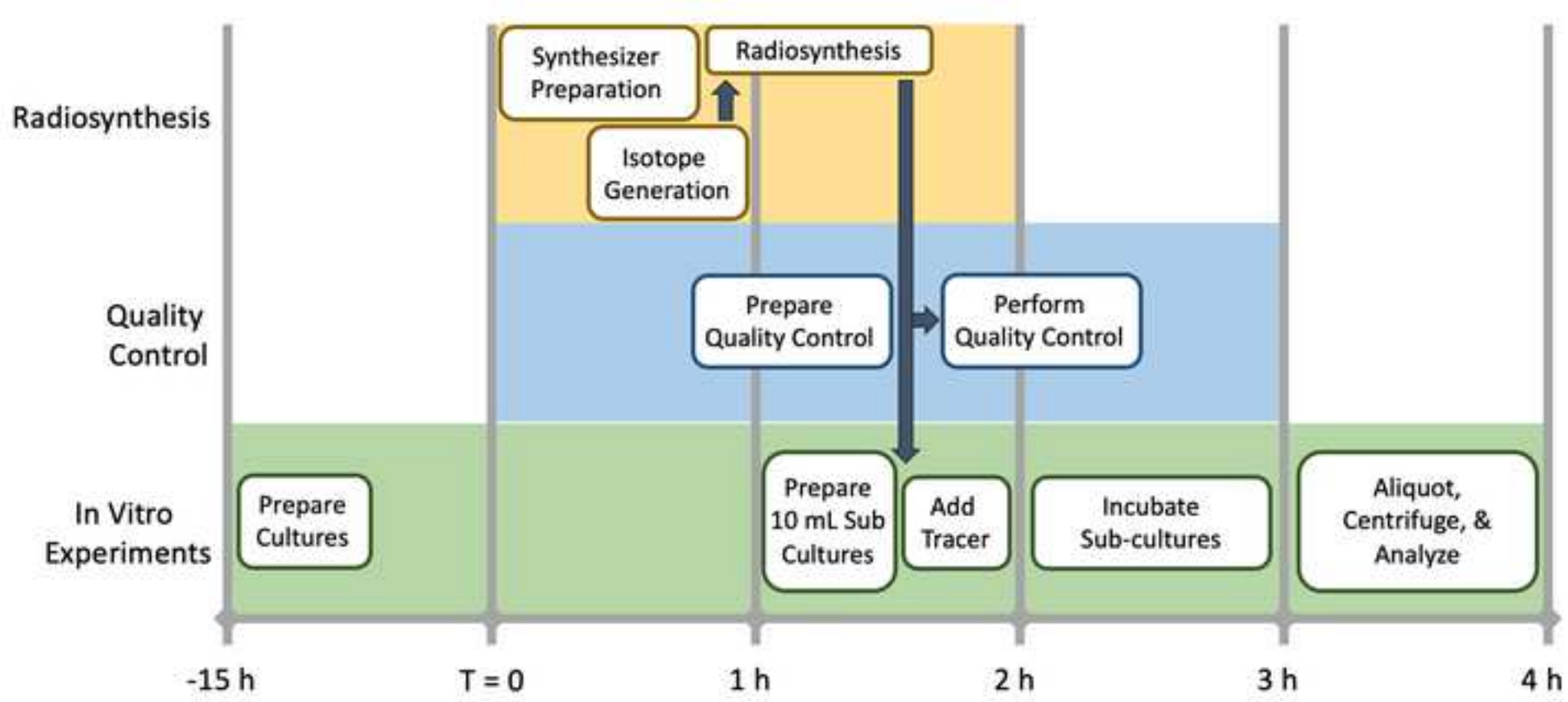


Figure 3

Click here to access/download;Figure;Figure 3.tif

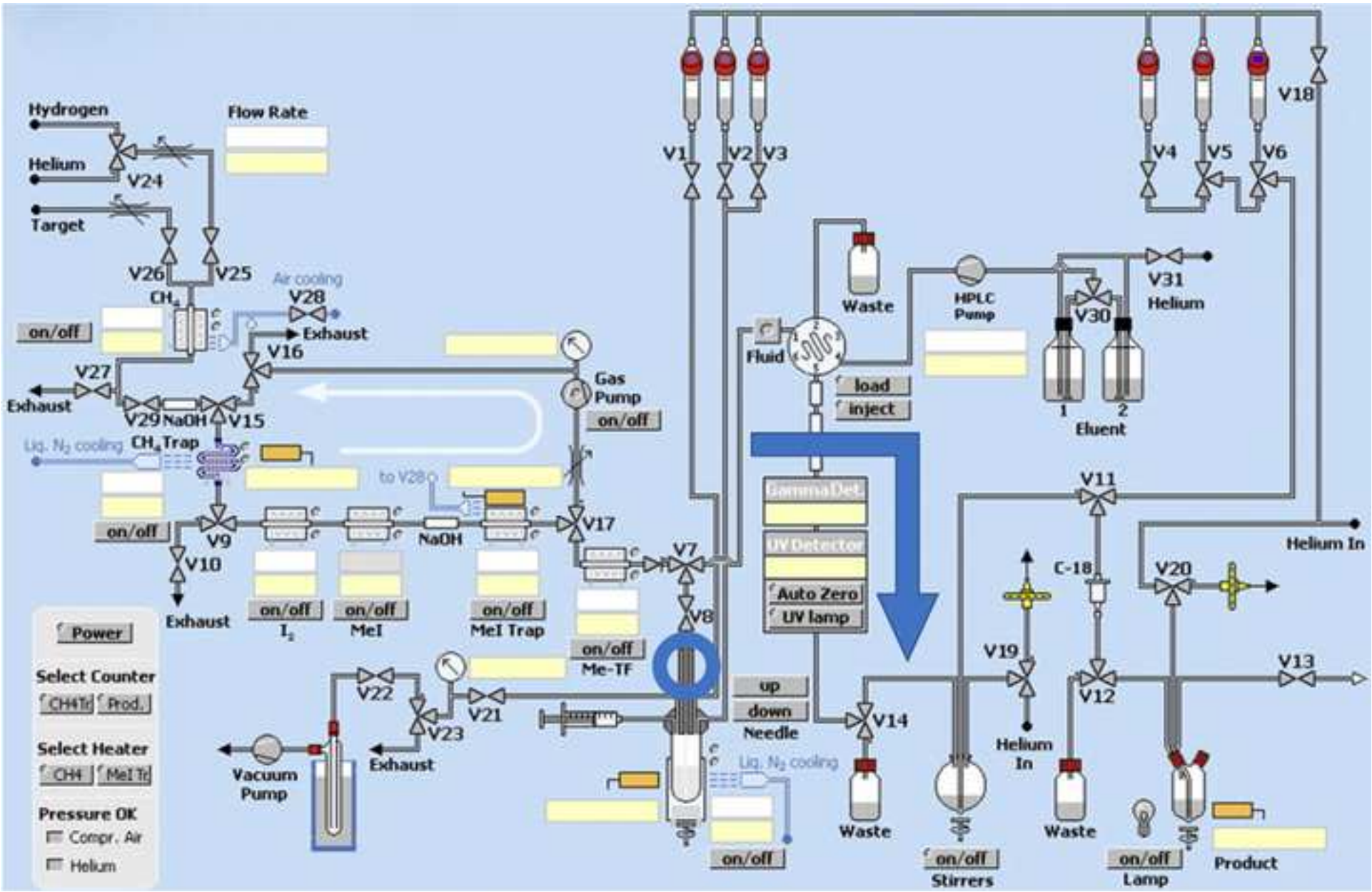
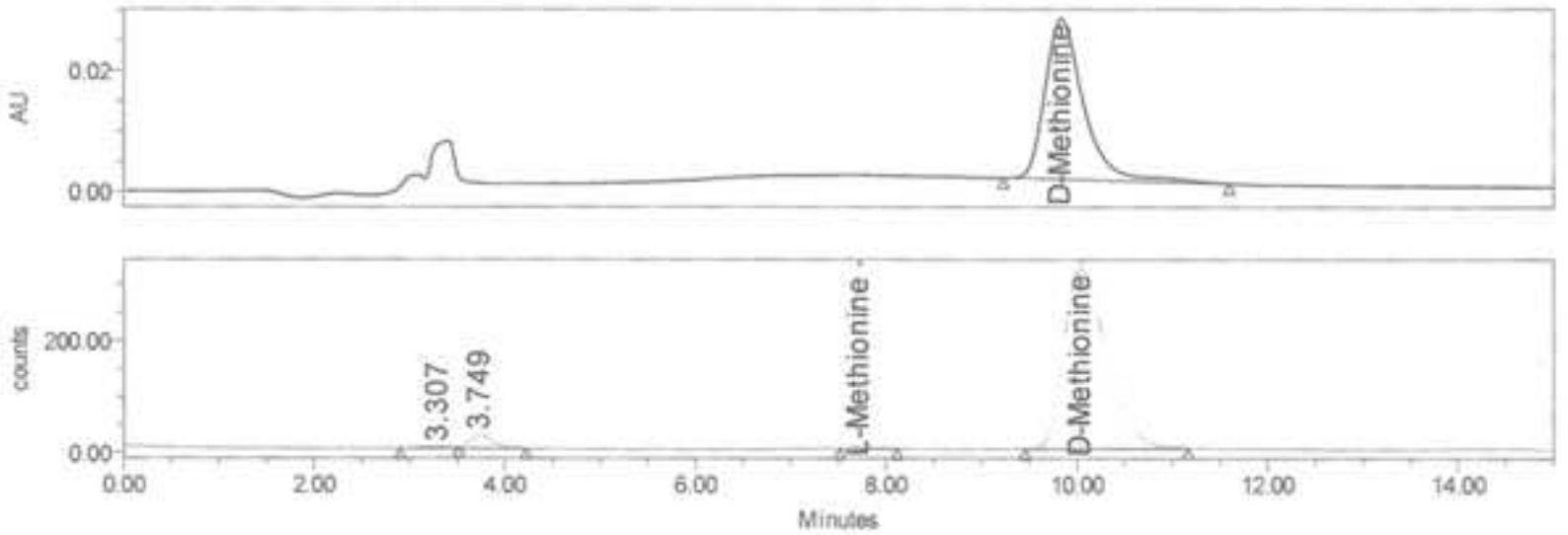
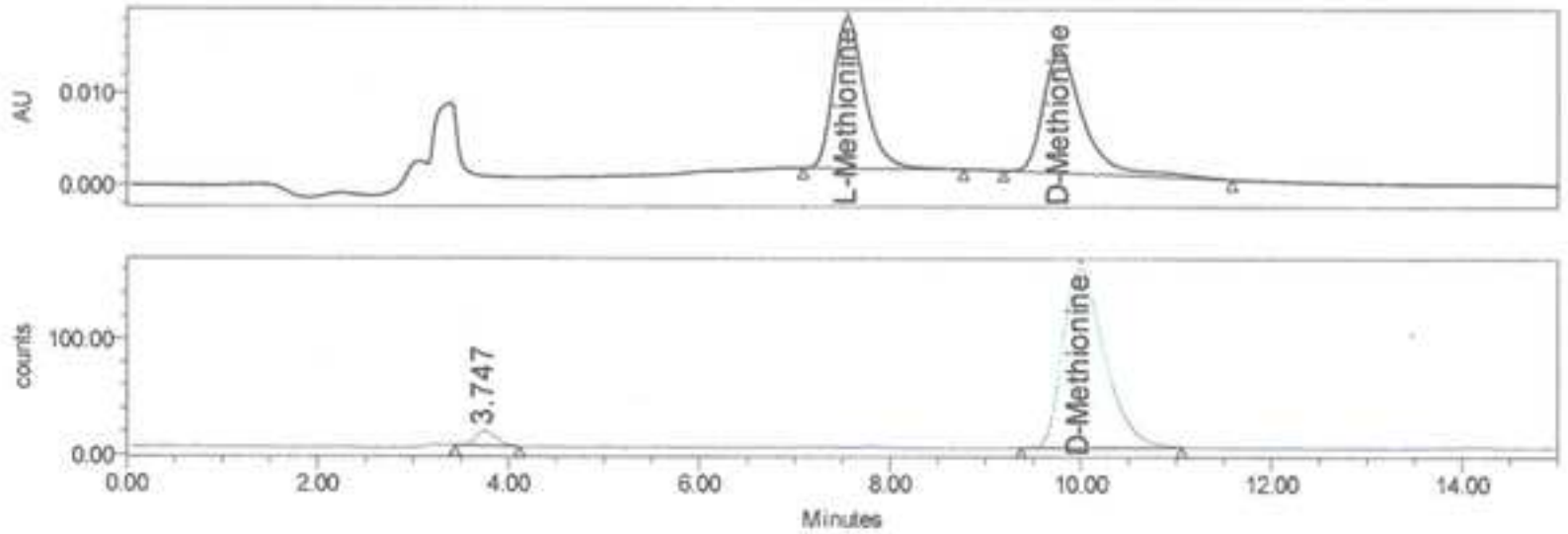


Figure 4



Channel: W2996 220.0nm-1.2; Processed Channel: W2996 PDA 220.0 nm at 1.2, Smoothed by 15 point Mean Filter.; Result Id: 1539; Processing Method: Methionine UV

Channel: eSATIN-Ch2; Processed Channel: Radioactivity, Smoothed by 9 point Mean Filter.; Result Id: 1540; Processing Method: Methionine Radio



Channel: W2996 220.0nm-1.2; Processed Channel: W2996 PDA 220.0 nm at 1.2, Smoothed by 15 point Mean Filter.; Result Id: 1498; Processing Method: Methionine UV

Channel: eSATIN-Ch2; Processed Channel: Radioactivity, Smoothed by 9 point Mean Filter.; Result Id: 1499; Processing Method: Methionine Radio

Figure 5

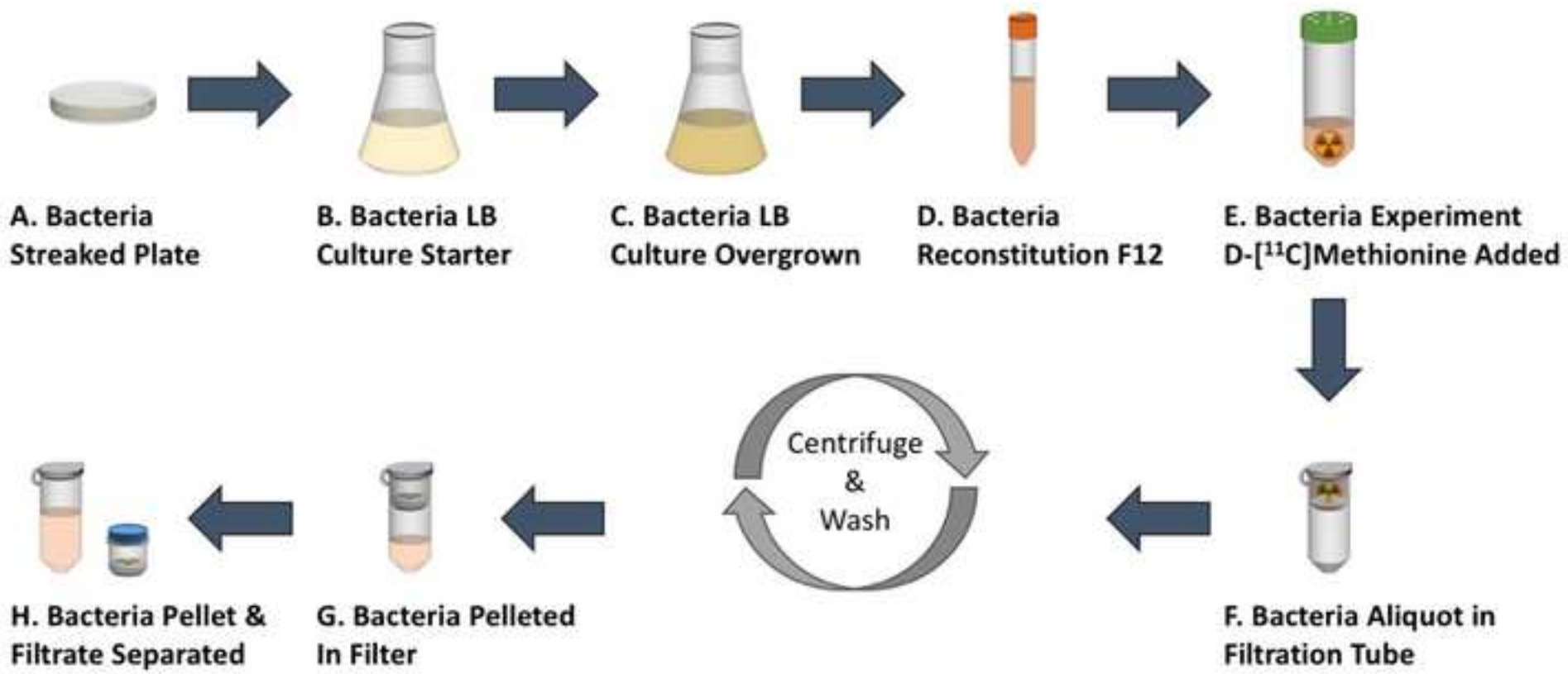
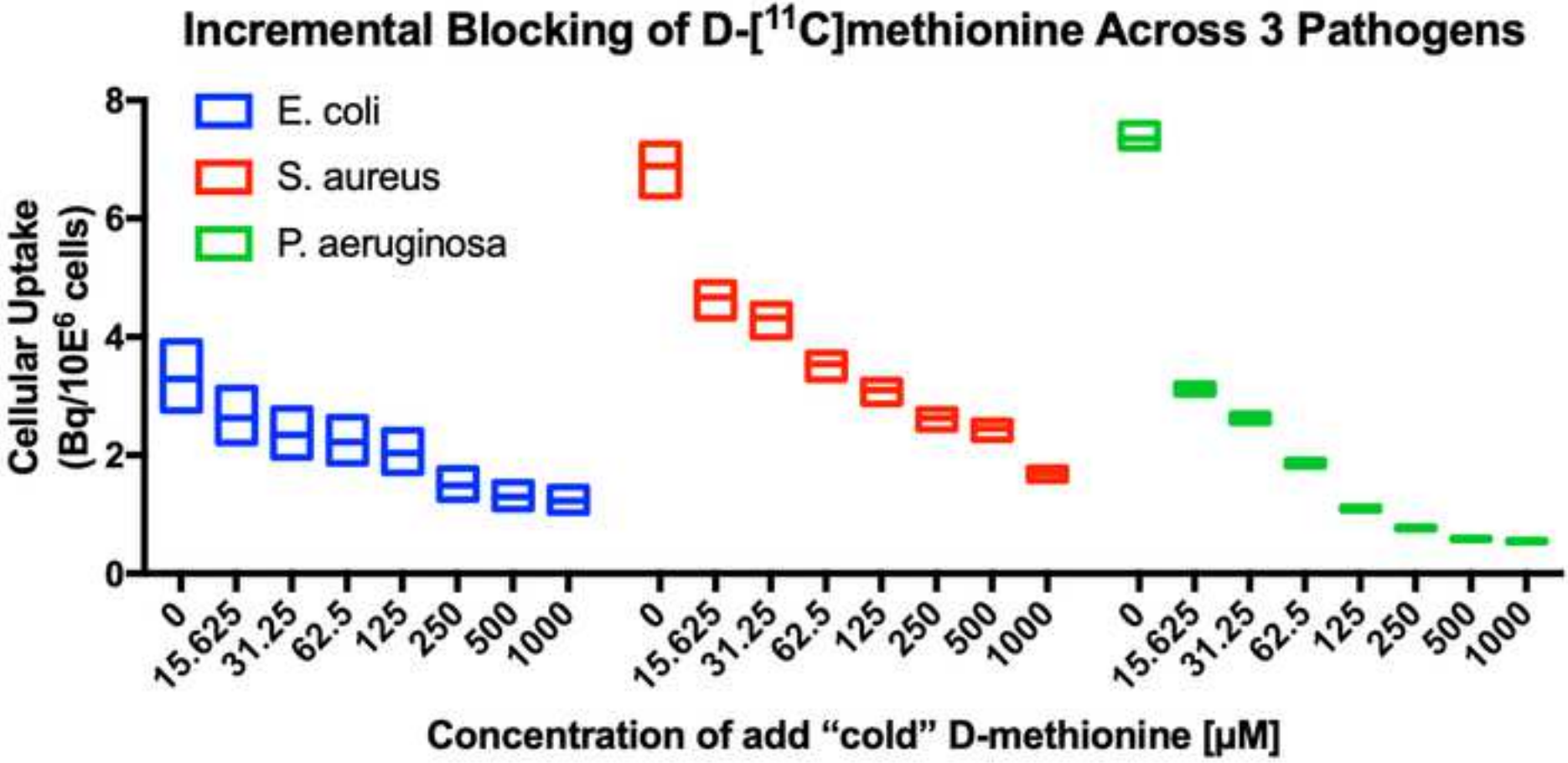


Figure 6



Name of Material/Equipment	Company	Catalog Number	Comments/Description
Materials and instruments for radiosynthesis of D-[^{11}C]methionine			
0.5 M NaOH	Sigma-Aldrich, St. Louis, MO, USA		
C18 Sep Pak	Waters, Milford, MA, USA		
D/L-Methionine	Sigma-Aldrich, St. Louis, MO, USA		Standards
D-Homocysteine	Sigma-Aldrich, St. Louis, MO, USA		Precursor
Ethanol	Sigma-Aldrich, St. Louis, MO, USA		
GE Medical Systems PET Trace	GE Healthcare, Uppsala Sweden		Cyclotron
Iodine	Merck, Darmstadt, Germany		
Nickel Catalyst	Shimadzu, Kyoto, Japan		
Nitrogen gas + 1% Oxygen	AirGas, Radnor, PA, USA		Target Gas
Saline	Sigma-Aldrich, St. Louis, MO, USA		
Sodium Hydrogen Phosphate	Sigma-Aldrich, St. Louis, MO, USA		

TRACERlab FXC	GE Healthcare, Uppsala Sweden		Synthesis Module
Water	Sigma-Aldrich, St. Louis, MO, USA		
Materials and instruments for quality control of D-[^{11}C]methionine			
Acetonitrile	Sigma-Aldrich, St. Louis, MO, USA		
Agilent 8890	Agilent, Santa Clara, CA, USA		Analytical GC
Bioscan AR2000	Bioscan, Santa Barbara, CA, USA		RadioTLC
Chirobiotic T2 column (250 x 4.6 mm)	Astec, Chattanooga, TN, USA		Chiral HPLC Column
Water	Sigma-Aldrich, St. Louis, MO, USA		
Waters 600 Controller	Waters, Milford, MA, USA		Analytical HPLC
Materials and instruments for bacterial uptake assays of D-[^{11}C]methionine			
15 mL Centrifuge Tubes	Corning, NY, USA		
50 mL Bio-Reaction Tubes	Celltreat, MA, USA		
E. Coli	ATCC, Manassa, VA, USA		25922
F12 Media	Thermo, Waltham, MA, USA		

Genesys 20	Thermo, Waltham, MA, USA		UV/Vis Spectrometer
Hidex AMG	Hidex, Turku, Finland		Gamma Counter
Innova 42	Brunswick, Lake Forest, IL, USA		Incubator/Shaker
LB Agar Plates	Teknova, Hollister, CA, USA		
LB Broth	Teknova, Hollister, CA, USA		
P. Aeruginosa	ATCC, Manassa, VA, USA		10145
S. Aureus	ATCC, Manassa, VA, USA		12600
Spin-X Filter Tubes	Corning, NY, USA		
UV Cuvettes	Fisher, Waltham, MA, USA		

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.

The manuscript has been proofread and all spelling and grammar issues resolved.

2. Please remove the embedded figure(s) from the manuscript. All figures should be uploaded separately to your Editorial Manager account. Each figure must be accompanied by a title and a description after the Representative Results of the manuscript text.

Each figure has been uploaded as a separate file.

3. Figure 1: Please use SI abbreviations for time: h instead of hr, etc.

The appropriate SI abbreviations have been installed.

4. Figure 2: Please remove the GE logo and branding.

The GE logo has been removed.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. 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.

All commercial language has been removed from the manuscript.

6. Please include a Summary that clearly describes the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"

A summary has been included.

7. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s).

All references numbers are displayed as superscripts.

8. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be"

throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

The protocol language has been updated to reflect the imperative tense.

9. Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).

The centrifuge speeds are now expressed as centrifugal force.

10. Please provide all experimental parameters. What are the parameters used for the gamma counter?

Experimental parameters for the gamma counter have been included

11. Please reference all figures in the manuscript text. Where is Figure 2 referenced?

Figure 2 is now referenced in the manuscript.

12. 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.

All essential steps in the protocol are highlighted.

13. 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.

All essential steps in the protocol are highlighted as specified.

14. Please discuss some limitations of the protocol in the Discussion.

Limitations in the protocol are discussed.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The manuscript by Parker et al a descriptive and informative report of the chemistry and in vitro use of 11C - Methionine. This will be helpful to newcomers to the field or for established investigators unused to working with very short-live nuclides.

Major Concerns:

1. The CGMP details in 2A are not relevant for preparation of the radiopharmaceutical for rapid in vitro studies. If they are required in the authors facility, a note saying this isn't necessary for the preclinical use could be included.

The CGMP details have been removed.

2. Is the DMet loop program something that comes with the module or was written in house? Need some description.

The loop program is adapted from a standard protocol of the module and a description is provided.

3. A schematic of the organic chemistry would be helpful.

A schematic of the chemistry has been included.

Minor Concerns:

4. All acronyms should be defined at first use (SWFI)

All acronyms are defined.

5. The sources for all materials and equipment should be given.

Sources for all equipment are provided.

6. Page 2: 11-carbon should be carbon-11 (also in some places it is capitalized and some places not)

The nomenclature for carbon-11 is consistent throughout the manuscript.

7. Page 3: radio tracer should be one word

This has been corrected.

8. Page 3: is 0.5M NaOH the final concentration of the solution or of the water used in 50%?

0.5 M NaOH is the concentration of the solution component so the final concentration of NaOH is 0.25M.

9. Page 4: vales should be valves

This has been corrected.

10. Page 6: net day should be next day

This has been corrected.

11. Page 5: It should be noted which type of cyclotron this is for. Many other machines

require a more manual start up. Might just say - start up cyclotron according to machine specific procedures.
This has been corrected.
12. Page 6: "needs to be within a designated" should be "need to be within a designated"
This has been corrected.
13. Page 7: milliCurie (µCi) - one of these is incorrect
This has been corrected.

Reviewer #2:
Manuscript Summary:
This methods paper describes the synthesis and QC of D-[11C]methionine. The radiochemistry is of basic level and the synthesis has been described before in ACS Infect. Dis. 2020, 6, 43− 49 by the same group. Besides, synthesis of L-[methyl-11C]-methionine is described elsewhere e.g. in J. Label Compd. Radiopharm 2008, 51 83-8). For education purposes, this paper can be useful.

Major Concerns:
None

Minor Concerns:
the correct phrasing is: D-[methyl-11C]-methionine.
This has been corrected.
Are dead bacteria used as a negative control?
Yes, D-[11C]methionine is a metabolic tracer so dead (non-metabolically active bacteria) are used as a control.
The curves of the bacterial uptake, how are these standardized as QC?
The uptake is reproducible across several experiments using different batches of radiotracer.
Why is the LB broth changed by Ham's F12 media?

The F12 media provides more consistent reproduction of behavior across multiple experiments executed on different days with different batches of radiotracer.

The optical density is used to convert to the concentration of colony-forming units (CFU/mL) for bacteria where ~1 OD600 corresponds to 8 x108 CFU/mL. This is not correct as you also measure the dead and non-replicating bacteria. CFU is determined after plating.

We routinely plate the bacteria from experiments to determine the CFU/mL to relate the OD with CFU.