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IPA Romania – Serbia

CROSS CARE

Joint working procedures
BOOKLET

Cross-border cooperation to foster the resilience of clinical management in cancer patients by establishing best practices in personalized molecular-based diagnostics, treatment, and long-term care

RORS00040

BETTER diagnosis, care, lives!

Spitalul Clinic Judetean de Urgenta „Pius Brinzeu" Timisoara
OncoGen Excellence Research Center

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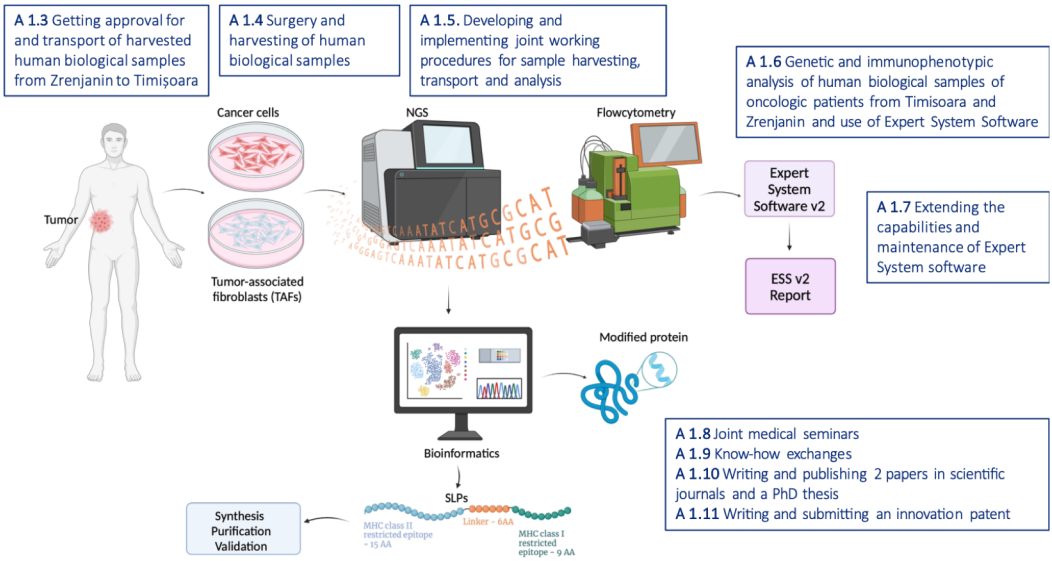
Project objective

Foster cooperation in Oncology in the cross-border region, with a focus on diagnostics and training, thus improving healthcare services to the benefit of oncologic patients' survival, life span and quality

How?

- Purchase of new medical equipment
- New joint procedures, know-how exchanges, and trainings



Results

- Set up a map of cancer genetic profile in the region
- Create a joint strategy for tackling cancer threats, benefiting the health system in both countries

Procedure for Conducting Activities within the CROSSCARE Project

As part of the IPA Interreg Cross-border Cooperation Programme between Serbia and Romania, we have been granted a project titled **"Cross-border Cooperation for Strengthening the Resilience of Clinical Management in Cancer Patients by Establishing Best Practices in Molecular Method-Based Personalized Diagnostics, Treatment, and Long-term Care,"** under the acronym **CROSSCARE**, Project Number **RORS00040**. The project is funded by **Interreg IPA Romania-Serbia Programme** and is being conducted in collaboration with the **Oncogen Research Center in Timișoara**.

The project involves collecting blood and tumor samples from patients with solid malignant tumors, which will be sent to Oncogen for genetic and molecular analysis. The goal is to provide personalized oncological therapy for each patient participating in the study and to identify a common biomarker in solid malignant tumors that could be targeted by a shared therapeutic agent. All patient-related procedures will be conducted in accordance with the principles of the Helsinki Declaration. The study will include all patients aged 18 and older who are eligible for blood and tumor sampling. Patients who pass away before sample collection or those who choose to withdraw from the study will be excluded.

Patients diagnosed with a malignant solid tumor in an outpatient or inpatient setting, or with a tumor of uncertain malignancy, will be admitted for inpatient treatment, either for surgical intervention or biopsy. Upon admission, they will sign standard documents, including:

- "Patient's Declaration of Consent for the Proposed Medical Procedure – Surgery"
- "Patient's Consent for Hospitalization"
- "Patient Information on Diagnosis and Treatment"
- Two additional declarations: one regarding compliance with hospital regulations and another granting consent for the use of video surveillance.

Patients will also be informed about the planned research study and, if they agree to participate, will sign an informed consent form. Each patient will then be assigned a unique code number, which will be used throughout the study and linked to their personal data, ensuring strict confidentiality. A record of these code numbers will be maintained, correlating them with the patient's name and surname.

Patients will undergo standard preoperative preparation, with an additional 4 mL blood sample collected in a tube containing an anticoagulant. The sample will be stored at room temperature. Once conditions are met for surgical treatment, the procedure will be performed under general, regional, or local anesthesia. Following tumor resection or biopsy, a tumor sample of at least 0.5 cm^3 will be collected, either as a single fragment or multiple fragments.

INFORMED CONSENT
For Participating in the Project

Project Name: "Cross-border cooperation to foster the resilience of clinical management in cancer patients by establishing best practices in personalized molecular-based diagnostics, treatment, and long term care", Project acronym: CROSSCARE, Project Number RORS00040, funded by the Interreg IPA Romania-Serbia Programme.

Dear Mr/Mrs,

You have been diagnosed with a malignant tumor, and you have been admitted to our institution for its operative treatment, or in the case of a biopsy, if your prescribing doctor so emphasizes.
Malignant tumor is a progressive disease, which arises on the basis of cell mutation of a certain organ of the body. Such cells are usually recognized and destroyed by the body's immune system, but sometimes the immune system fails, tumor cells multiply and a tumor is formed. Unlike benign tumors, malignant tumors are independent, develop to the detriment of the organism and have the possibility of colonizing other organs, i.e. they can metastasize. This disease can be successfully treated if it is diagnosed in the initial stages. In the later stages, certain drugs, operative procedures and radiological methods can slow down the disease, rarely stop it, and sometimes it can even go back when it is possible to remove the tumor entirely by surgery. Despite the progress of medicine and the existing methods of diagnosis and treatment, malignant tumors are still demanding treatment, often with a poor prognosis, a severe course of the disease with a fatal outcome. Prognoses are individual, depending on the patient, type of tumor, time of diagnosis and available treatment methods.
Your treatment plan is to remove the tumor, to send it for further pathohistological analysis in order to determine the best possible treatment method in cooperation with oncologists, radiologists and other necessary specialties. Of course, while waiting for the results of the tumor analysis, the entire team of doctors, nurses and other medical and non-medical personnel is in charge of your speedy recovery and treatment in your best interest.

As medicine develops, so do our possibilities for your most successful treatment. For this reason, we applied for the IPA project that we named with acronim CROSSCARE. The project implies cross-border cooperation, funded by the European Union, and which aims to treat malignant, solid (not blood) tumors as efficiently as possible through cooperation with the Oncogen research center in Timisoara. The project was approved by the European Commission as the best project proposal.

The project envisages that a sample of your blood and tumor will be sent to the Oncogene Research Center in Timişoara, for further cellular, molecular and genetic analysis. Analyzes are performed in order to obtain the best possible oncology therapy adapted to you personally and to determine a common target on all solid malignant tumors, on which a common agent would act. Data about you and your disease will be entered into a common registry, developed within the project, in order to more easily monitor and analyze malignant solid tumors.

The study will be conducted according to all the principles of the Helsinki declaration.

All personal information are strictly confidential.

You can withdraw from the study whenever you want, even though you sign to participate in the study and the project, without any consequences for your treatment.

You can discuss all information with your surgeon or medical members of the project team - Vanja Kunkin MD, Marija Djujić MD and Rastislav Filko MD.

You can learn more about IPA projects, the Oncogen research center and the project itself at the following links:
https://europa.rs/ipa-fondovi/; https://oncogen.ro/ro/; https://bolnica.org.rs/

Patient data	
Family name	First name
Date of birth	Telephone
Address	

I agree to participate in the above-mentioned project!

Date **Patient signature**

I was informed in detail by the informant about the nature of my illness and the way to treat it, as well as that an additional blood sample and a part of the tumor removed from me will be taken in order to be sent to the Oncogene research center in Timisoara for further molecular and genetic analysis. Everything is aimed at getting as personal and better postoperative therapy as possible.

It is clear to me that the benefit of the study is great, both for me and for other patients with solid (non-blood) tumors.

I am aware that the data about my illness will be entered into a common register in order to better monitor patients with similar illnesses, and that my personal data will be fully protected.

I am aware that all possible measures will be taken to prevent the occurrence of adverse effects during blood sampling or that the best possible treatment will be implemented in the event of adverse effects occurring during and after blood sampling.

All collected personal data will be strictly confidential and will not be publicly available.

I agree that my contact information will be used in the future to monitor my recovery and inform about the obtained results and proposed further therapy, as well as for the exchange of information needed during the conduct of the study.

I am aware that I can discuss any unknown data or ambiguities with the attending physician or members of the project team.

I voluntarily agree to participate in the mentioned study of sampling my blood and a part of the tumor for molecular and genetic analysis, with the aim of obtaining personalized therapy, determining a common therapeutic goal for solid (non-blood) tumors and entering data about me and my disease in the community registry.

Also, I am informed that despite having signed the consent to participate in the study, I can refuse to participate in the study at any time without consequences for my further treatment.

Date

Signature

INFORMED CONSENT

Dear Patient,

You are invited to participate in a medical research study investigating precision diagnostics and immunotherapies applicable to neoplastic diseases, as part of the CROSSCARE project (*Cross-border cooperation to foster the resilience of clinical management in cancer patients by establishing best practices in personalized molecular-based diagnostics, treatment, and long-term care, RORS00040*). This study is conducted by the OncoGen Center within the "Pius Brînzeu" Emergency Clinical County Hospital, Timișoara.

For the purpose of this research, 4 mL of venous blood will be collected during the blood donation process, using sterile, single-use kits. Before the sample is taken, a clinical examination will be performed, and throughout the procedure, you will be monitored by qualified medical personnel.

Potential Risks and Adverse Effects

The risks associated with blood donation are minimal but may include:

- Dizziness, in which case you will be monitored until the symptoms subside.
- Hematoma formation, caused by vein perforation and blood leakage into the surrounding tissues; if this occurs, the area will be compressed with a sterile cold compress, and an ointment will be applied to promote blood reabsorption.
- Infection at the venipuncture site, in which case you will receive appropriate treatment.

Additionally, following the surgical resection procedure, tumor fragments (with a maximum volume of 5 mm^3) will be collected via biopsy puncture from the excised tissue. This procedure will not affect your therapeutic management or diagnosis.

Your participation in this clinical study is voluntary. You will not receive direct benefits from participating in this medical research. However, the indirect benefits include the optimization of immunologic treatment techniques for cancer, which will benefit future oncology patients in both public and private healthcare institutions.

All scientific data obtained will remain strictly confidential. Your identity will not be disclosed in any documents related to the research activity. Instead, a numerical code (ID donor) will be used, generated according to the standard procedures for the donation of human-derived products.
The collected blood and tissue samples will not be stored; they will be used exclusively for scientific purposes and, upon completion of the research, they will be disposed of in accordance with the current regulations on biological waste of human origin.

I, the undersigned, ……., hereby give my consent in accordance with the provisions of Order No. 386/07.04.2004 of the Ministry of Health, regarding the approval of the Implementation Rules of the Patient Rights Law No. 46/2003.
For further information regarding this medical research, you may contact the OncoGen Medical Research Center, within the "Pius Brînzeu" Emergency Clinical County Hospital, Timișoara.
I certify that I have read, understood, and fully accept the above information, and as a result, I sign this informed consent.
I have received an original copy of this consent form, while another original copy remains with the study coordinator - Emergency Clinical County Hospital "Pius Brînzeu" - OncoGen.

Patient's name
Patient's signature
Date

JOINT INFORMED CONSENT FORM

Patient Information and Participation in the CROSSCARE Project

Dear Patient,

You have been diagnosed with a malignant solid tumor, and you have been admitted to our institution for surgical treatment. The planned therapeutic approach involves tumor resection, followed by pathohistological analysis to determine the most appropriate treatment strategy in collaboration with a multidisciplinary team, including oncologists, radiologists, and other relevant specialists.

While awaiting the results of the tumor analysis, our medical team - including physicians, nurses, and support staff - will provide comprehensive post-operative care, ensuring your optimal recovery and well-being. Advances in medical research continue to enhance treatment options, and we strive to integrate the latest scientific developments into clinical practice for the best possible patient outcomes.

As part of this effort, our institution is participating in the CROSSCARE Project (RORS00040) - a cross-border initiative funded by the European Union. This project aims to enhance the management of malignant solid tumors through collaboration between the Emergency Clinical County Hospital "Pius Brînzeu" Timișoara – OncoGen and General Hospital "Đorđe Joanović" Zrenjanin. The project has been recognized by the European Commission as an exemplary initiative in personalized molecular-based diagnostics, treatment, and long-term care.

As part of this project, prior to your surgery, 4 mL of blood will be collected under sterile conditions, along with a tumor sample obtained during the surgical procedure. These biological samples will be transported under strictly regulated conditions to the designated research center (OncoGen) for molecular and genetic analysis.

The results of these analyses will allow for the development of a personalized therapeutic approach, tailored specifically to your tumor's molecular profile. The aim of this precision medicine strategy is to:

- Optimize your post-surgical treatment by identifying the most effective targeted therapy.
- Prevent tumor recurrence and metastasis or, where applicable, stabilize the disease.
- Advance cancer research by identifying common molecular targets across different tumor types, potentially leading to novel therapeutic strategies applicable to multiple solid tumors.

To facilitate long-term patient monitoring and improve healthcare strategies, your medical data will be securely stored in a dedicated patient registry, created as part of this project. All personal information will be handled in compliance with data protection regulations, ensuring complete confidentiality.

By consenting to blood and tumor sample collection, you contribute not only to your own treatment optimization but also to global cancer research efforts aimed at developing more effective therapies. The knowledge gained through this project could have a significant impact on future cancer treatments worldwide.

Participation in this study does not pose additional health risks, as:

- Blood collection (4 mL) is part of the routine preoperative preparation.
- Tumor tissue collection does not alter the standard surgical procedure or impact your subsequent treatment.

Possible risks associated with blood sampling are minimal but may include:

- Local complications, such as minor bleeding, bruising, hematoma formation, or inflammation at the puncture site.
- Rare complications, including vascular injury, allergic reactions (local or systemic), or retention of foreign material.

All necessary precautionary measures will be taken to minimize these risks, and any complications will be managed in accordance with best medical practices.

By participating in this study, you have the opportunity to enhance your own treatment while also contributing to groundbreaking research in oncology. Your involvement in this project could help pave the way for new treatment strategies that benefit cancer patients globally.

If you agree to participate, please let our medical team know. Should you have any questions or concerns, we are available to provide further information. You can discuss all information with your surgeon or medical members of the project team: Vanja Kunkin, MD and Marija Djujić, MD - General Hospital "Đorđe Joanović" Zrenjanin; Tudor Popoiu, MD and Florina Bojin, MD, PhD - Emergency Clinical County Hospital "Pius Brînzeu" Timișoara - OncoGen.

Thank you for your willingness to contribute to the advancement of cancer research and treatment.

Sincerely,
Medical team
Emergency Clinical County Hospital "Pius Brînzeu" Timișoara
General Hospital "Đorđe Joanović" Zrenjanin

Protocol for Blood Samples Collecting

Project Name: “Cross-border cooperation to foster the resilience of clinical management in cancer patients by establishing best practices in personalized molecular-based diagnostics, treatment, and long term care”, Project acronym: CROSSCARE, Project Number RORS00040, funded by the Interreg IPA Romania-Serbia Programme.

Upon admission of the patient, and its identity checks, as part of the preoperative preparation, which also includes blood sampling, 4 ml of blood will be taken for analysis within the study, so no additional procedure will be performed, unless it is necessary for technical or medical reasons. The blood will be collected in a tube with an anticoagulant. Blood collection will be done according to the institution's current procedure (01-837/142). Sampling will be done by the responsible nurse in the ward where the patient is placed. Sampling will be performed by the responsible nurse in the ward where the patient is placed, under the supervision of a member of the project team. The test tube is provided according to the needs of the project. After pouring the sample into it, it will be closed and marked with a previously determined code number, which the patient receives at the reception.

The blood sample will then be stored in the transporter at room temperature. When the conditions are met, when it has been verified that the appropriate samples have been packed, the transporter is placed in a transport vehicle and the transport to the Oncogen Research Center in Timisoara, Romania begins. During transportation, the sample must be kept at room temperature, protected from impacts and possible breakage and spillage. Transport will be carried out by an authorized person of the General Hospital Đorđe Joanović Zrenjanin. In order for biological material to be transported across the border, a permit for the transport of biological material across the border, which is obtained from the Ministry of Health of Serbia, will first be secured. Upon arrival at Oncogen, the sample will be handed over to the responsible person and further prepared for the necessary analyses.

Protocol for Blood Sample Collection in Oncologic Patients

Objective:

To safely and accurately collect 4 mL of venous blood from oncologic patients with solid tumors, using EDTA anticoagulant tubes, while maintaining aseptic technique and ensuring patient comfort. This protocol is established for the CROSSCARE project (Cross-border cooperation to foster the resilience of clinical management in cancer patients by establishing best practices in personalized molecular-based diagnostics, treatment, and long-term care, RORS00040) – Emergency Clinical Hospital Pius Brinzeu Timisoara.

1. Preparation and patient identification

- Perform hand hygiene according to hospital infection control protocols and wear appropriate personal protective equipment (PPE) (gloves, lab coat, and face mask if necessary);
- Identify the patient using two identifiers (e.g., full name and date of birth) and verify with the laboratory request form;
- Confirm that the patient is an oncologic patient with a solid tumor, ensuring eligibility for sample collection under the study or clinical procedure;
- Explain the procedure, emphasizing its importance in monitoring or diagnosing their condition. Obtain written consent.

2. Preparation of equipment

- Sterile gloves
- Alcohol swabs (70% isopropyl alcohol or chlorhexidine)
- Tourniquet
- Sterile single-use needle (21G or 23G butterfly for fragile veins)
- Vacuum blood collection system (or syringe if required)
- 4 mL EDTA (Ethylene-Diamine-Tetraacetic Acid) tube (purple-top tube)
- Sterile gauze and adhesive bandage
- Sharps disposal container

3. Venous puncture site selection and preparation

- Position the patient in a comfortable, seated, or semi-reclined position to prevent vasovagal reactions.
- Extend the patient's arm, preferably using the antecubital fossa veins (median cubital, cephalic, or basilic). If peripheral veins are difficult to access, consider using a butterfly needle for smaller veins.
- Apply the tourniquet approximately 3–4 cm above the venipuncture site, ensuring it is tight but not obstructing arterial flow.
- Palpate the vein and cleanse the area with an alcohol swab, applying friction in a circular motion from the center outward. Allow the area to air dry completely to avoid contamination.

4. Blood collection

- Anchor the vein by stretching the skin taut with the non-dominant hand.
- Insert the sterile needle at a 15–30° angle, bevel facing up, into the vein.
- If using a vacuum collection system, attach the 4 mL EDTA tube (purple top) and allow it to fill by vacuum pressure. If using a syringe, gently pull back the plunger to collect 4 mL of blood.
- Do not excessively manipulate the syringe plunger, as this may cause hemolysis and affect test results.
- Once blood collection is complete, release the tourniquet before removing the needle to prevent hematoma formation.

5. Post-collection handling

- Withdraw the needle smoothly and immediately apply sterile gauze over the puncture site.
- Ask the patient to apply gentle pressure for 30–60 seconds, then secure with an adhesive bandage.
- Immediately dispose of the used needle in a designated sharps container to prevent needlestick injuries.

6. Sample labeling and documentation

- Invert the EDTA tube gently 8–10 times to ensure proper mixing with the anticoagulant and prevent clot formation.

- Label the tube at the bedside, ensuring the following information is included: Patient's full name; Date of birth; Hospital ID number; Date and time of collection; Collector's initials
- Cross-check the labeled tube against the laboratory request form to confirm accuracy.

7. Final steps

- Ensure the patient is feeling well before allowing them to leave. Instruct them to report any signs of dizziness, bruising, or excessive bleeding.
- Discard all used materials properly following biohazard waste disposal protocols.
- Remove gloves and perform hand hygiene.
- Transport the sample to the laboratory within 72 hours at room temperature (18–25°C) to prevent sample degradation.

Conclusion

This protocol ensures the safe, standardized, and aseptic collection of 4 mL of venous blood in EDTA tubes from oncologic patients with solid tumors, within the CROSSCARE project (Cross-border cooperation to foster the resilience of clinical management in cancer patients by establishing best practices in personalized molecular-based diagnostics, treatment, and long-term care, RORS00040). Proper labeling, handling, and transport are crucial for sample integrity, ensuring reliable diagnostic or research outcomes.

References:

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Working Procedure for Peripheral Blood Harvesting

Project Name: “Cross-border cooperation to foster the resilience of clinical management in cancer patients by establishing best practices in personalized molecular-based diagnostics, treatment, and long term care”, Project acronym: CROSSCARE, Project Number RORS00040, funded by the Interreg IPA Romania-Serbia Programme.

Tumor is performed according to the valid procedure of the General Hospital Đorđe Joanović Zrenjanin (01-837/142), which is adapted for the needs of the study.

- Blood is sampled as part of standard blood sampling for preoperative preparation.
- The patient should rest for 15-30 minutes before blood sampling.
- Check the identity of the patient and stick the previously assigned code number on the test tube.
- Introduce yourself to the patient and explain the blood sampling procedure and why an additional amount of blood is being taken for the purposes of the study, for which the sampler needs to make sure that he has given his consent.
- Prepare the necessary equipment for blood sampling.
- Wash your hands according to the instructions for washing your hands, disinfect them and put on disposable gloves.
- The patient is placed in a supine position.
- Blood must not be removed from the hematoma.
- Blood must not be drawn from the arm in which the infusion was administered.
- Blood must not be drawn from a vein containing a cannula or an arteriovenous fistula without a physician's consent.
- Place the curtain 10-15 cm above the puncture site, ask the patient to make a fist, choose a vein and puncture site, evaluate by palpation which vein is sufficiently filled with blood, and possibly check the veins on the other arm.
- Disinfect the chosen injection site with ethanol and wait for the site to dry or dry it with sterile gauze.
- Screw the selected needle into the needle holder.
- Remove the protective cap from the needle and insert the tip of the needle into the vein at an angle of 10-20°.

- The direction of the needle follows the direction of the vein.
- After taking blood for standard checks as part of preoperative preparation, sample blood in the test tube intended for the purposes of the study.
- Close the test tube and perform homogenization by turning the test tube with the cap down and returning it to its original position. Place the test tube in the test tube rack.
- Use the curtain for no longer than 1 minute.
- Ask the patient to relax the hand.
- After removing the last vacuum tube, cover the needle with a sterile swab.
- Pull out the needle with a quick movement and only then press the venipuncture site. Give clear instructions to the patient to hold the swab tightly for the next 15 minutes.
- Dispose of the used needle, on which the protective cap is not returned, in the yellow container.
- Dispose of used swabs and gloves in a yellow bag for infectious waste.
- The sample is stored at room temperature until transport.

Working Procedure for Venous Peripheral Blood Harvesting

Objective:

To collect 4 mL of venous peripheral blood from participants using EDTA as an anticoagulant for the clinical study within the CROSSCARE project (Cross-border cooperation to foster the resilience of clinical management in cancer patients by establishing best practices in personalized molecular-based diagnostics, treatment, and long-term care, RORS00040).

Materials needed:

- **EDTA Tubes**: 4 mL capacity, with EDTA anticoagulant
- **Needles**: 21-23 gauge, sterile
- **Syringes**: 5 mL, sterile (optional)
- **Tourniquet**: For venous occlusion
- **Alcohol Swabs**: For skin disinfection
- **Gloves**: Sterile, for handling blood samples
- **Labeling Materials**: For sample identification

Procedure:

Preparation:

- Ensure all necessary materials are available and ready.
- Wear sterile gloves throughout the procedure.

Participant Preparation:

- Explain the procedure to the participant and obtain informed consent
- Ensure the participant is seated comfortably with their arm extended and supported

Venous puncture:

- Apply a tourniquet to the upper arm to occlude venous flow
- Clean the venipuncture site with an alcohol swab
- Perform venipuncture using a sterile needle and syringe (if using)
- Insert the needle into a suitable vein (e.g., median cubital vein)

Blood Collection:

- If using a syringe, slowly draw 4 mL of blood into the syringe

- Alternatively, attach an EDTA tube to the needle and allow blood to flow directly into the tube until it reaches the 4 mL mark
- Release the tourniquet once blood flow begins

Sample Handling:

- Immediately mix the blood with the EDTA anticoagulant by gently inverting the tube several times
- Label the tube with the participant's ID and date/time of collection
- Store the sample at room temperature or refrigerate at 4°C

Post-Collection:

- Apply gentle pressure to the venipuncture site with a cotton ball or gauze until bleeding stops
- Dispose of needles and other sharps in a biohazard container

Transportation and Storage:

- Transport samples to the laboratory in a secure, insulated container to maintain temperature stability
- Process samples according to the study protocol for further analysis

Safety Precautions:

Always handle blood samples with caution to prevent exposure
Ensure proper disposal of biohazardous materials

This procedure is designed to ensure safe and effective collection of peripheral blood samples for activities within the CROSSCARE project (Cross-border cooperation to foster the resilience of clinical management in cancer patients by establishing best practices in personalized molecular-based diagnostics, treatment, and long-term care, RORS00040). Always follow local regulations and guidelines for handling biological samples.

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Protocol for Selecting and Surgical Removal of Tumor Samples to be Analysed within the Project

Objective:

To choose the appropriate patient and adequately obtain the samples needed for the realization of the CROSSCARE project (Cross-border cooperation to foster the resilience of clinical management in cancer patients by establishing best practices in personalized molecular-based diagnostics, treatment, and long-term care, RORS00040) – Emergency Clinical Hospital Pius Brinzeu Timisoara.

1. Patient selecting:

- patients with proven or highly suspected solid malignant tumors, which were diagnosed by various imaging methods
- patients older than 18 years, both gender
- patients who can undergo the intervention under local or general anesthesia
- the patient must be alive at the time of sampling, a sample cannot be taken from a cadaver
- patients who die after the intervention in any period will certainly be processed in terms of processing blood and tumor samples

2. Tumor sampling:

- patients can be biopsied or operated on in the ambulatory, daily hospital or inpatient treatment in regional or general anesthesia.
- patients who are inoperable and/or do not have a certain diagnosis, and malignancy is highly suspected, undergo a biopsy
- patients with a pathohistologically confirmed malignant solid tumor, which can be surgically removed, are prepared for surgery and undergo a resection procedure. Of course, if they have no contraindications for such a procedure
- patients who do not have pathohistologically confirmed malignancy, and are suspected of malignancy, can be operated on immediately if there are medical indications for it
- the sample obtained by biopsy can be multifragmented or from one piece. The total volume should be about 0.5 cm^3.

- The samples obtained after tumor resection are obtained by excising a part of the tumor, with a total volume of about 0.5 cm^3. If it is not possible otherwise, in some procedures, it is possible to make a multifragmentary sample with a total volume of up to 0.5 cm^3.
- Sampling must be performed under sterile conditions.
- The samples are then inserted into a sterile bottle and covered with a sterile saline solution of antibiotics and antifungals. The solution must exceed the upper edge of the sample by at least 1 cm.
- The closed bottle is then stored at a temperature of 4-8 C° until transportation to the OncoGen.

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Working Procedure for Fresh Tumor Tissue Harvesting

Project Name: “Cross-border cooperation to foster the resilience of clinical management in cancer patients by establishing best practices in personalized molecular-based diagnostics, treatment, and long term care”, Project acronym: CROSSCARE, Project Number RORS00040, funded by the Interreg IPA Romania-Serbia Programme.

Tumor is performed according to the valid procedure of the General Hospital Đorđe Joanović Zrenjanin (01-1695/2, amended under number 01-95/47), which is adapted for the needs of the study.

- The patient is identified.
- Prepare a sticker with the code number previously assigned to the patient for the purposes of the study.
- Prepare a container for the tumor sample, which must be sterile and empty.
- If the tumor is completely removed with a sterile scalpel and under sterile conditions, a part of the tumor, which is not smaller than 0.5 cm^3, is removed. If a biopsy is performed, the sample should be of the same size if it is single-part or at least a total volume of 0.5 cm if it is multifragmentary. Then, under sterile conditions, it is inserted into a previously prepared container for biopsies.
- The prepared solution of saline solution, antibiotics and antimycotics is then poured into the container so that it is at least 1 cm above the upper edge of the sample.
- The container is closed and marked with a previously prepared sticker with the patient's code number.
- The sample is stored at room temperature until transport.

Working Procedure for Fresh Tumor Tissue Harvesting

Objective:
To obtain fresh cells from resected tumors by performing a biopsy puncture and transporting the samples in a sterile solution to another facility for further processing, for the clinical study within the CROSSCARE project (Cross-border cooperation to foster the resilience of clinical management in cancer patients by establishing best practices in personalized molecular-based diagnostics, treatment, and long-term care, RORS00040).

Materials needed:
- **Sterile Biopsy Needle**: 18-20 gauge
- **Syringe**: 5-10 mL, sterile
- **PBS Solution**: Phosphate-buffered saline
- **Antibiotics/Anti-fungal Solution**: Appropriate concentrations of antibiotics (e.g., penicillin-streptomycin) and anti-fungal agents (e.g., amphotericin B)
- **Sterile tubes**: 50 ml Falcon tubes with tight-fitting lids for sample transport
- **Gloves**: Sterile, for handling samples
- **Labeling Materials**: for sample identification

Procedure
Preparation:
- Ensure all necessary materials are available and ready
- Wear sterile gloves throughout the procedure.

Tumor Preparation:
- Obtain the resected tumor specimen and place it on a sterile surface
- Identify the most representative areas of the tumor for biopsy.

Biopsy Puncture:
- Perform a biopsy puncture using a sterile needle and syringe
- Insert the needle into the tumor tissue and aspirate a small sample
- Repeat as necessary to obtain sufficient material (no more than 5 mm^3)

Sample Handling:
- Immediately transfer the biopsy sample to a sterile tube containing PBS with antibiotics and anti-fungal agents
- Ensure the tube is filled to an appropriate level to cover the sample completely
- Gently mix the contents to distribute the sample evenly

Labeling and Storage:
- Label the tube with the patient's ID, date, and time of collection
- Store the tube at room temperature (approximately 20-25°C) during transport to another facility (OncoGen)

Transportation:
- Transport the sample in a secure, insulated container to maintain temperature stability
- Ensure the tube is kept upright and protected from physical damage during transport

Post-Transport Processing:
- Upon arrival at the destination facility (OncoGen), the sample is immediately process to obtain fresh cells
- Follow established protocols for cell isolation and culture

Safety Precautions:
- Always handle biological samples with caution to prevent exposure
- Ensure proper disposal of biohazardous materials.

Quality Control:
- Verify the integrity of the samples upon arrival at the destination facility
- Monitor sample handling and processing to ensure compliance with protocols.

This procedure is designed to ensure safe and effective collection and transportation of tumor biopsy samples for further analysis, within the CROSSCARE project (Cross-border cooperation to foster the resilience of clinical management in cancer patients by establishing best practices in personalized molecular-based diagnostics, treatment, and long-term care, RORS00040). Always follow local regulations and guidelines for handling biological samples.

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https://www.aafp.org/pubs/afp/issues/2002/0315/p1155.html
https://pmc.ncbi.nlm.nih.gov/articles/PMC9327433/
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https://geiselmed.dartmouth.edu/radiology/wp-content/uploads/sites/47/2019/04/Anatomically_guided_Core_Needle_Biopsy_of_Bone_Tumors.pdf

Protocol for Transport of Biological Samples

Objective:

To develop a procedure by which blood and tumor samples will be transported in the best and safest way from the General Hospital Đorđe Joanović Zrenjanin to the OncoGen Excellence Research Center in Timisoara, within the framework of the cross-border cooperation project is one of the goals of the CROSSCARE project (Cross-border cooperation to foster the resilience of clinical management in cancer patients by establishing best practices in personalized molecular-based diagnostics, treatment, and long-term care, RORS00040) – Emergency Clinical Hospital Pius Brinzeu Timisoara.

1. Blood and tumor samples are marked with a code number for each patient immediately after sampling. The code number represents the serial number of the patient's admission and serves to identify the patient and protect his privacy.
2. Before and after sampling, a notification letter is filled out by members of the project team, which contains all the relevant data about the patient and his illness, and at the same time, data privacy rights are protected.
3. After sampling, the blood is stored at room temperature, and the tumor tissue in the refrigerator at a temperature of 4-8 °C. Care is taken to ensure that the samples do not turn over.
4. During that time, documentation is being prepared for transfer across the border, which includes customs and shipping documentation, in Serbia for export, and in Romania for import.
5. The time from sampling to preparation for analysis must not be longer than 72 hours
6. When all the administrative conditions for sending are met, the samples are prepared for transport.
7. The blood is packed in a transport case with a guaranteed temperature of 4-8 °C and with fasteners in order to prevent overturning and breaking of the packaging.
8. Tumor samples are prepared in another transport case, where a temperature of 20-25 °C is ensured and a fixture is provided to prevent overturning and breaking of the packaging.

9. Then the transport cases are placed in the vehicle intended for transport and measures are taken to secure them, so that they do not tip over.
10. The car is driven by an authorized person of the General Hospital Đorđe Joanović Zrenjanin, who can be a driver of the same institution or one of the members of the institution's project team, who have the necessary valid documentation, such as a driver's license and a valid passport. In the vehicle there may be one or more persons, the driver and/or team members and/or persons authorized by the project manager. Authorization is obtained orally or in writing. The person can also be authorized by another team member.
11. The transport starts after providing all the necessary documentation and technical conditions by the vehicle and passengers. The time of departure is coordinated with the possibilities of receiving samples at the OncoGen Center
12. Upon arrival at the border, the necessary documents for customs and shipping are submitted both on the Serbian and Romanian sides.
13. After crossing the border, the samples are taken to Timis county customs, and then to the Oncogen center for receiving and processing the samples (must be written by the Romanian side)

References

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https://www.dropoff.com/blog/everything-you-need-to-know-about-transporting-blood-and-specimens/
https://www.ouh.nhs.uk/biochemistry/tests/documents/transport-and-storage.pdf
https://www.dhl.com/discover/en-at/industry-insights/life-science-healthcare/biologische-proben-und-herausforderungen-beim-transport

Working Protocol for Immunophenotypic Analysis of Sanguine Nucleated Cells

Reagents and consumables	Equipments
PBS	Laminar floow hood
Ficoll	Centrifuge
DMSO	Micopipettes
RPMI	Cryobox
FCS/FBS	Ultrafreezer -80° C
Antibiotic solution Pen/Strep	Flowcytometer
Glycerol 40%	
Venous peripheral blood (EDTA, citrate, heparine)	
Cryogenic vials	
Sterile gloves	
Pasteur pipettes	
Falcon tubes 50 ml	
Fluorescent-labelled antibodies	

1. Dilute the blood sample 1 : 1 with sterile PBS at room temperature (e.g.: 2 ml blood + 2 ml PBS; 4 ml blood + 4 ml PBS) in a 50 ml Falcon tube and mix well by pipetting
2. Place in a 50 ml Falcon tube Ficoll (at room temperature) in a volume equal to the volume of the undiluted blood sample (e.g.: for 2 ml blood - 2 ml Ficoll; for 4 ml blood - 4 ml Ficoll)
3. Pipette the diluted blood sample into the Ficoll tube, carefully, on top, by tilting the Ficoll tube at an angle of approximately 45°, so that the entire amount of blood is above the Ficoll layer
4. Centrifuge the Ficoll tube + diluted blood at 2500 rpm for 30 minutes, acceleration 3, deceleration 0 (without brake); the time required for centrifugation will be approximately 45-50 minutes
5. After centrifugation, the tube will be placed in the hood in a suitable support, in a vertical position, to visualize the layers of components: at the bottom – erythrocytes and platelets, granulocytes; Ficoll; mononuclear cell ring (PMBC); plasma
6. Using a Pasteur pipette, the mononuclear cell ring will be collected from the Ficoll-plasma interface, which will be pipetted into another 50 ml Falcon tube; the total volume of cells should not exceed 2 ml (initial blood samples of 2 ml) or 4 ml (initial blood samples of 4 ml)

7. Add a double volume of PBS to the Falcon tube with mononuclear cells and wash by centrifugation at 1500 rpm for 10 minutes, acceleration 9 (max.), deceleration 9 (max)
8. After washing, discard the supernatant and resuspend the sediment (pellet) in a volume of 1.8 ml in the following medium (warm, at 37° C): RPMI + 10% FCS/FBS + 1% Pen/Strep
9. Transfer the 1.8 ml volume to a cryogenic tube and add 200 µl DMSO (10% of the total volume); mix the contents of the tube quickly by rotating/inverting and place in the cryobox for long-term freezing, initially at -80° C, subsequently at -196° C (liquid nitrogen)

Between steps 7 and 8 of the above protocol, the erythrocytes will be isolated and frozen as follows:

- Using a Pasteur pipette, the following layers remaining after harvesting the mononuclear cell ring will be removed: plasma and Ficoll; the bottom layer, composed predominantly of erythrocytes (RBCs), will remain
- From an initial blood volume of 2 ml, we will obtain 1 ml of RBC; from 4 ml of initial blood we will obtain approximately 2 ml of RBCs
- Add an equal volume of glycerol 40% over the volume of RBCs (1, respectively 2 ml, depending on the initial blood volume), mix well by pipetting and transfer to cryogenic tubes (1 tube – if the initial volume of the blood sample was 2 ml; 2 tubes – if the initial volume of the blood sample was 4 ml)
- Insert the tube/tubes into the cryobox and wait for the completion of the procedure for isolating PBMCs, to insert the cryobox into the deep freezer, at -80° C

Cryogenic tube labeling

- All cryogenic tubes will have the patient code, consisting of the initials of the name and the sample number from the Sample Reception register within the CrossCare project (e.g.: XY_15)
- Cryogenic tubes for PBMCs will be labeled as follows: patient code + PBMCs (e.g.: XY_15_PBMCs)
- Cryogenic tubes for RBCs will be labeled as follows: patient code + RBCs + tube no. (when there are 2 tubes of RBCs)(e.g.: XY_15_RBCs_1 or XY_15_RBCs_2)
- The date on which the products obtained from the venous blood samples were frozen will be noted on all tubes

- All this information (no. of tubes of each cell type, date of freezing, etc.) will also be noted in the CrossCare project register

Labeling examples:

XY_15_PBMCs	XY_15_RBCs_1	XY_15_RBCs_2
27.03.2025	27.03.2025	27.03.2025

Immunophenotypic analysis

Sample Preparation

- Aliquot 100 µL of well-mixed whole blood into a flow cytometry tube.
- Add surface antibodies according to the panel (typically 5-10 µL per antibody, following manufacturer's instructions).
- Mix gently and incubate for 15-20 minutes at room temperature (in the dark).

RBC Lysis

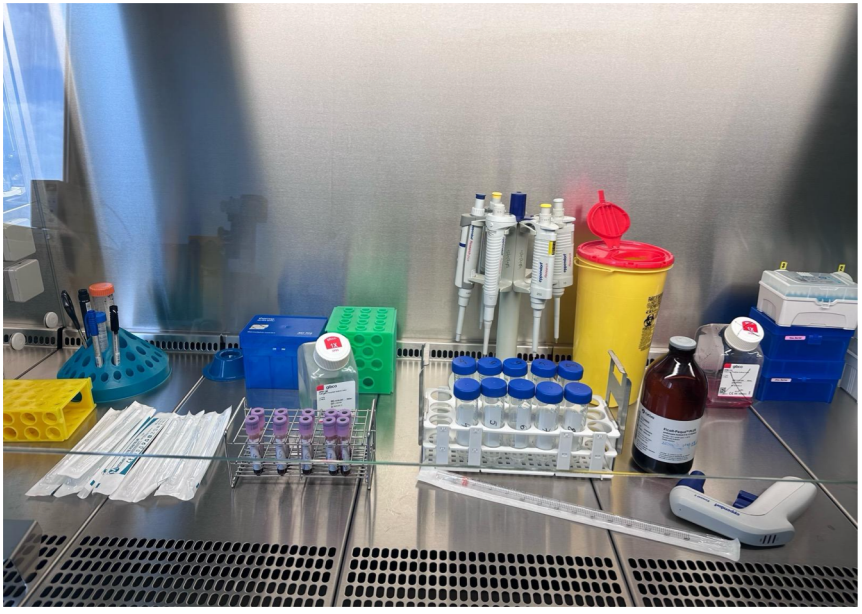
- Add 2 mL of RBC lysis buffer (if ammonium chloride-based, incubate for 10 minutes; if commercial, follow instructions)
- Vortex briefly and incubate in the dark
- Wash with 2 mL PBS (with optional 1% FBS/BSA)
- Centrifuge at 300-400g for 5 minutes, discard supernatant
- Resuspend the cell pellet in 300-500 µL PBS

Acquisition and analysis

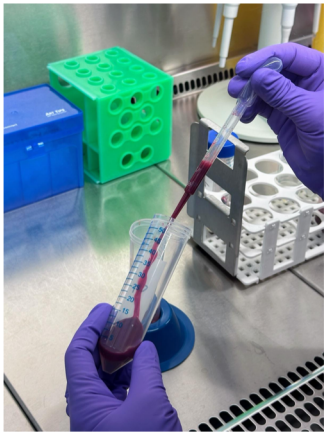
- Acquire data using a flow cytometer set for **minimum 50,000 events** per tube (adjust based on expected cell populations)
- Use appropriate compensation controls (single-stained tubes or beads)
- Analyze the cellular populations based on fluorescence emission using **FlowJo** or **FCS Express** software

Marker	Purpose
CD45	Pan-leukocyte marker (gating reference)
CD3, CD4, CD8	T cell subsets
CD19, CD20	B cell markers
CD56, CD16	NK cells
CD14, CD64	Monocytes
HLA-DR	Activation marker
CD34, CD117	Stem/progenitor cells
CD38, CD138	Plasma cells
CD33, CD13, CD15	Myeloid markers

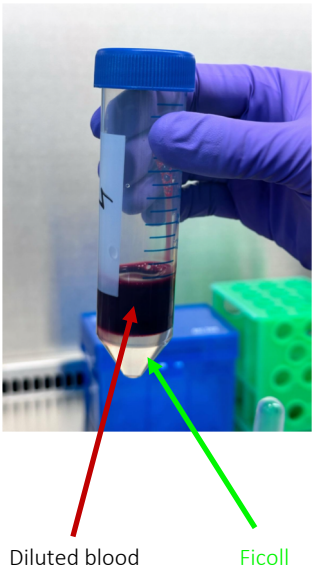
Cell culture laboratory - OncoGen

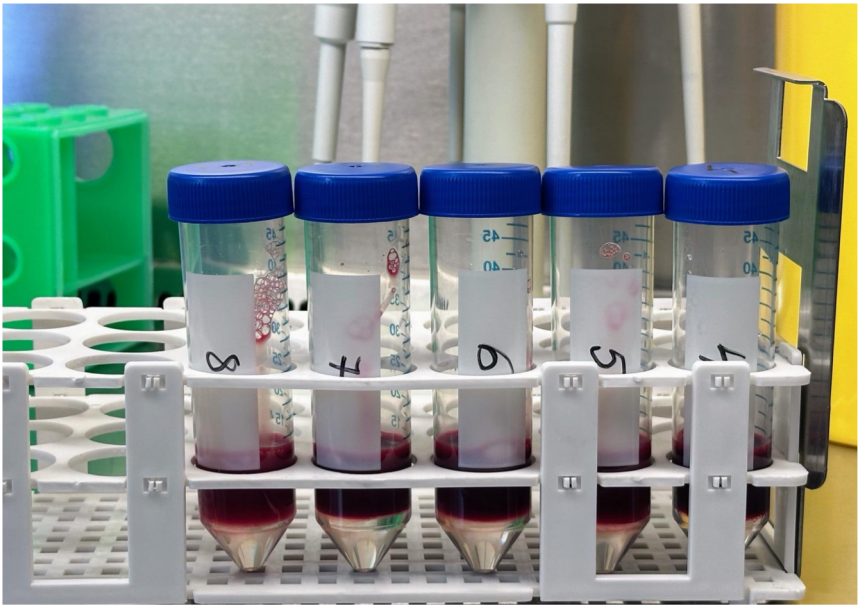


Samples, consumables and reagents required for isolation of PBMCs from venous peripheral blood

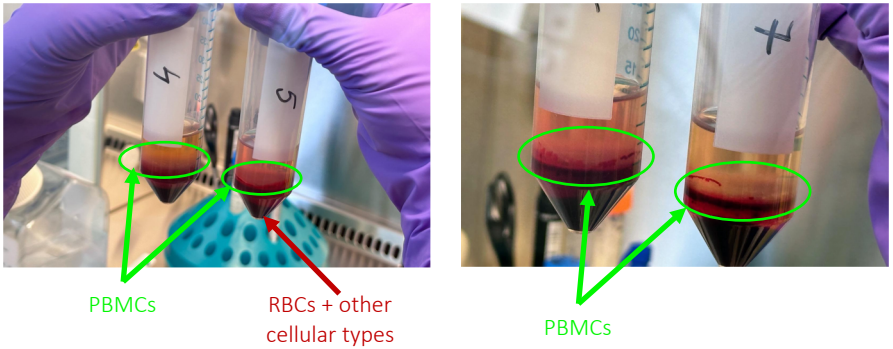


The diluted blood is carefully pipetted onto the Ficoll layer by tilting the tube at approximately 45°, ensuring that the entire volume of blood remains above the Ficoll layer.

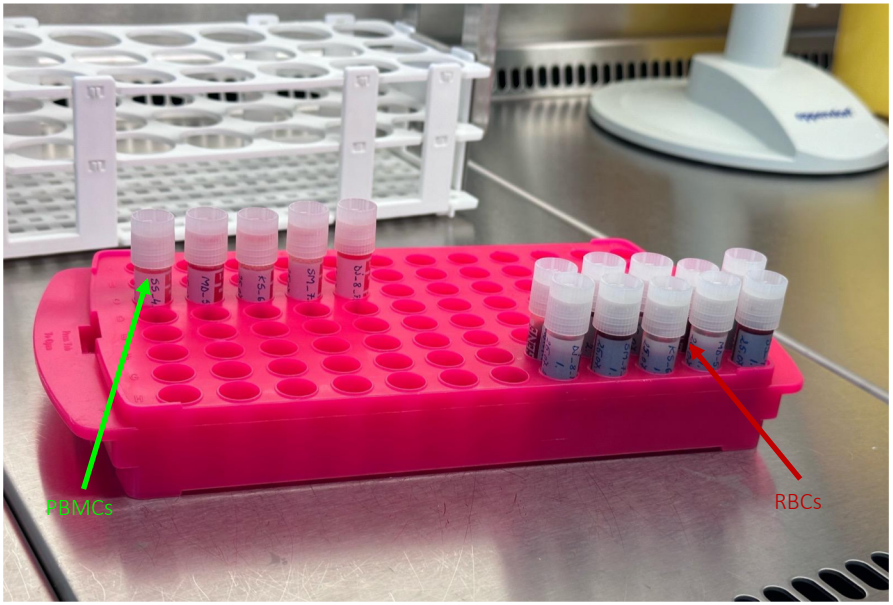




Appearance of five peripheral venous blood samples layered on top of the Ficoll layer, prior to processing according to the subsequent steps of the protocol.



Representative appearance of the peripheral blood layers after centrifugation, with distinct plasma, PBMC, Ficoll, and RBC + platelet layers.



Cryogenic vials containing PBMCs and RBCs. PBMCs are cryopreserved in RPMI supplemented with FCS and 10% DMSO in 2 mL cryogenic vials (one vial per blood sample). RBCs are cryopreserved in 20% glycerol in two vials per blood sample. The vials are initially stored in cryoboxes at −80 °C and subsequently transferred to liquid nitrogen for long-term storage.

Cytek Northern Lights Workflow
Start-up, Daily QC, Shutdown

1. Start-up

Cytek NL has a 30 min warm up time

- Power an the cytometer → windows password
- Open the SpectroFlo software→Log in

Run water during warm up

- Acquisition→Default
- Remove the water tube for the sample probe(when changing sample tubes, wait until the SIT flush is complete!!)
- Load a tube with 2.5 ml DI water
- Select the flow rate (high)
- Click Start and run water (as the cytometer warms up, aprox.25 min)

2. Daily QC

Prepare SpectroFlo QC beads

- Vortex beads bottle
- Add 1 drop beads in to 300 µl in the same solution used for the sheat solution on the instrument, in a 5 ml tube
- Vortex tube

Click on the QC tab

- Load the 5 ml bead tube on the cytometer
- Check bead lot

→ Always select the correct bead lot when performing Daily QC.

→ Different bead lots have different fluorescence intensities

→ Each time open a new lot number of SpectroFlo beads, must import the bead lot ID into the library

→ Bead lot files can be downloaded from the Resources section cytekbio

- Click Start Acquisition

→ QC will take 5 min

→ Once Acquisition is complete, two SIT Flushes are automatically performed to clear the beads from the sample line

→ Daily QC Passes→Message is displayed „Daily QC has completed and Passed”

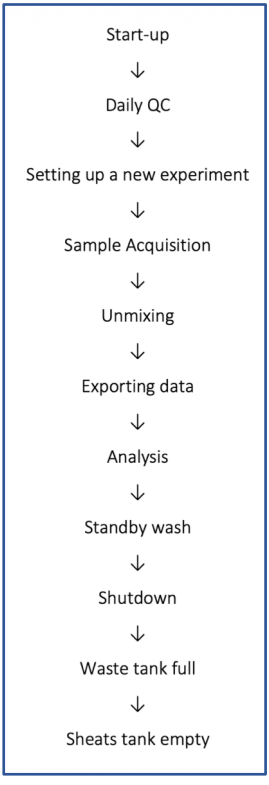
- Return bead bottle and diluted beads solution to 2-8^{0}C, protect from light. Reuse diluted beads solution for up to 5 days (need ≥80 µl for a QC run)

3. Shutdown

The procedure takes approximately 5 minutes
The procedure flushes the flow cell and sample lines
Cytometer Menu → QC & Setup or Acquisition → Fluidics Shutdown

- load a tube containing 3 ml of 10% bleach solution(prepared by adding 1 part household bleach to 9 parts DI water) on the SIP → Continue
- load a tube containing 3 ml of DI water → Continue
- load a tube containing 3 ml of 50% Contrad 70 → Continue
- load a tube containing 3 ml of DI water →Continue
- after procedure, to complete → Done
- exit SpectroFlo software
- turn off the cytometer and workstation
- the SIP must be submerged in the DI water at the end of the procedure

Cytek Northern Lights Workflow



Cytek Northern Lights Spectral Flowcytometer Immunophenotyping Protocol

1. Start-up

- start workstation and flow cytometer (top left side button)
- start software
- login in
- wait for flow cytometer connection (green tick √ cytometer display monitor)
- flush the tube → wait for maintenance completion for the tube positioned at SIP→OK
- position a new tube with 300 µl DI water at SIP → start → record
- purchase from the tube with DI water during warm up lasers
- warm up lasers - 30 min (green tick √ after 30 min)

2. QC controls & beads

- QC setup
- check current batch QC beads
- prepare QC tube (vortex for 5 seconds QC beads vial, put 1 drop in the tube marked QC beads, add 300 µl DI water, vortex again for 5 seconds)
- position QC beads tube at SIP immediately after vortexing → start
- passed QC(green tick √)

3. Panel

- CD45 PerCP-Cy5.5 (1µl/sample)
- CD3 PerCP (1µl/sample)
- CD4 FITC (1µl/sample)
- CD8a PE-Cy5 (1µl/sample)
- CD19 PE (1µl/sample)
- CD56 APC (5µl/sample)

4. Steps

- determine the required volume of each antibody to be pipetted for the antibody mixture
- determine the sample volume to be pipetted
- pipette each antibody, considering the number of samples present in the experiment (e.g. 4 samples → 4x1µl CD4 FITC) into the tube marked for the antibody mixture
- visually inspect the tip after each pipetting to ensure the presence of antibodies

- pipette the antibodies as low as possible, in the center of the tube, avoiding their dispersion on the tube walls
- mark the tubes for the experiment, 2 tubes for each sample (unstained and MC)
- pipette the antibody mixture into the multicolor tubes, as low as possible, in the center of the tubes, avoiding dispersion of the antibody mixture on tube walls
- pipette the blood sample (100 µl) into each tube (unstained and MC), let the blood drip from the tip as low as possible, in the center of the tube, over the antibody mixture
- leave the tubes at room temperature, in the dark, for 30 minutes, for Ab binding
- prepare lysis solution 1:10 (1 ml Lysing Solution + 9 ml distilled water)
- pipette 2 ml lysis solution into each tube, vortex, leave at room temperature, in the dark, for 20 minutes
- centrifuge the tubes at 500 x g / 5min / 21°C
- decant the supernatant in a single movement, avoid shaking and rotating the tube to avoid entraining antibodies at the bottom of the tube (contains cell debris + lysed erythrocytes)
- add 1 ml PBS to each tube (cell washing), centrifuge at 500 x g / 5min / 21°C
- remove the supernatant and resuspend the cells in 500 µl PBS
- vortex the tubes and acquire the data using spectral flowcytometer

5. Acquisition mode

- unstained all tubes (tube1/tube 2/…) → unmixing → MC tubes (MC tube 1/MC tube 2/…)
- unstained tube 1/MC tube 1/Unstained tube 2/MC tube 2/… → Unmixing

!The Unmixing button (tab up) becomes active only after acquiring all unstained tubes in the experiment!

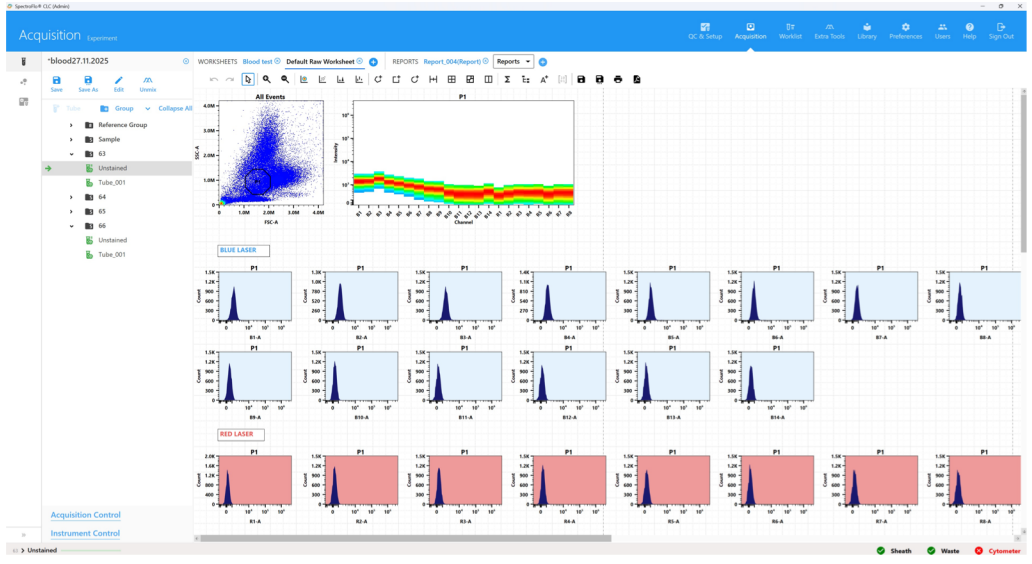
6. Export FCS files

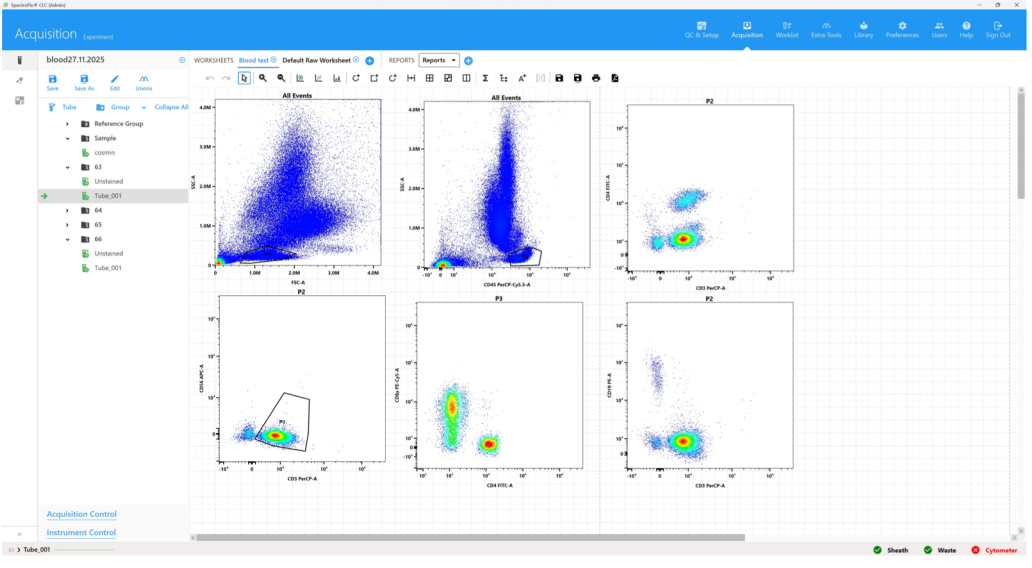
- close the experiment
- click My Experiment
- select the experiment → click Export →choose a Directory → click Export

7. Reuse Single Color Controls

- acquisition module → open My Experiments → right click on the saved experiment → click Duplicate
- all Reference Controls and Multicolor data are preserved
- marker labels may need to be added for new sample groups and tubes as needed
- export FSC files

Example of peripheral blood immunophenotyping



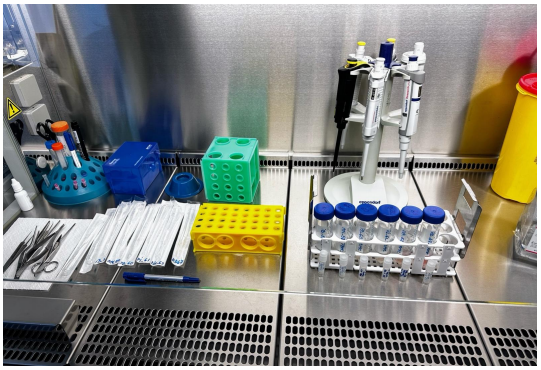


Cytek Northern Lights Spectral Flowcytometer Immunophenotyping Analysis Protocol

Thawing and freezing of PBMCs

1. Set a water bath at 37°C
2. Place the PBMCs tubes into the water bath to partially thaw (80%)
3. Transfer the cellular suspension into a 50 ml Falcon tube
4. Wash by centrifugation at 1500 rpm for 10 min. in warm medium (RPMI 1640 + 10% FCS +1% Pen/Strep)
5. Resuspend the pellet in 1 ml PBS
6. ***Take 400 µl of cellular suspension and split it in 2 tubes for further flowcytometric analysis**
7. Wash again the remaining of the cells in 2 ml PBS
8. Centrifuge at 1500 rpm for 10 min.
9. Resuspend the pellet in 1.8 ml warm medium (RPMI 1640 + 10% FCS +1% Pen/Strep)
10. Transfer the cellular suspension in cryogenic vials
11. Add 200 µl DMSO and place the tubes in a cryobox for storage at -80°C (short time); -196°C (long term storage)

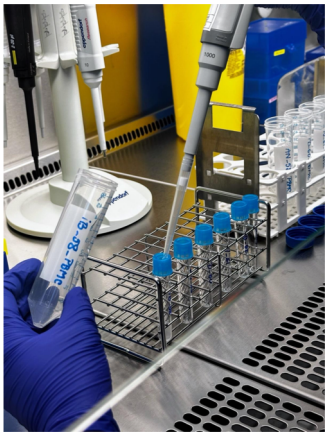
Reagents and consumables	Equipments
Blood	Centrifuge
Isolated PBMCs	Vortex
5 ml tubes	Micropipettes
Pipettes tips	Cytek spectral flowcytometer
Fluorochrome-conjugated antibodies	
Sterile gloves	
PBS (Phosphate Buffered Saline)	
Lysis solution	
RPMI 1640 +10% FCS +1% Pen/Strep	
DMSO (Dimethyl Sulphoxide)	
PBS (Phosphate Buffered Saline)	



Cellular suspension transferred into 50 ml falcon tubes

Addition of warm culture medium (10 ml)

Centrifugation, 10 minutes, 1500 rpm, rt

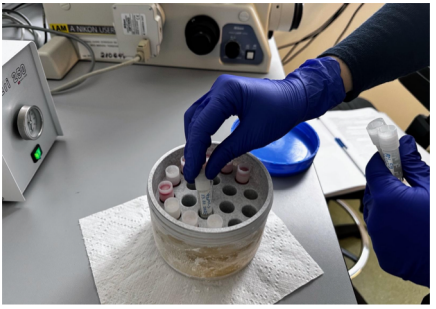


Additional step

Filtration of cellular suspension through 40 µm pore size mesh to avoid agglutination

Transfer of 200 µl cellular suspension in the 5 ml flowcytometry tubes (the antibodies were added in the multicolor tubes)

Resuspend the remaining cells in 1.8 ml culture media (RPMI + 10% FCS + 1% P/S); Add 200 µl DMSO



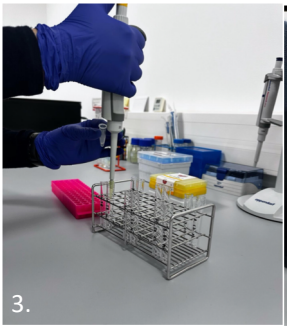
Place the tubes in the cryobox and store it at -80°C

Labelling of PBMCs for flowcytometry

1. For each PBMCs sample, the flowcytometric analysis requires 2 tubes of 5 ml: 1 tube containing unstained cells (control) and 1 tube containing the cells + the fluorochrome-conjugated specific antibodies
2. We used the following antibodies panels for staining and identification of PBMCs populations:

Panel 1	Panel 2
CD45 FITC	CD45 PerCP-Cy 5.5
CD3 APC-H7	CD3 PerCP
CD4 PerCP-Cy 5.5	CD4 FITC
CD8 PE-Cy 5	CD8 PE-Cy 5
CD19 PE	CD19 PE
CD56 APC	CD56 APC

3. Add the antibodies cocktail in the MC tube containing 200 µl PBMCs
4. Vortex well the mixture
5. Incubate at room temperature for 20 min. in the dark
6. Wash by centrifugation at 1500 rpm for 5 min.
7. Resuspend the pellet in 500 µl PBS
8. Vortex well and the samples are ready for acquisition

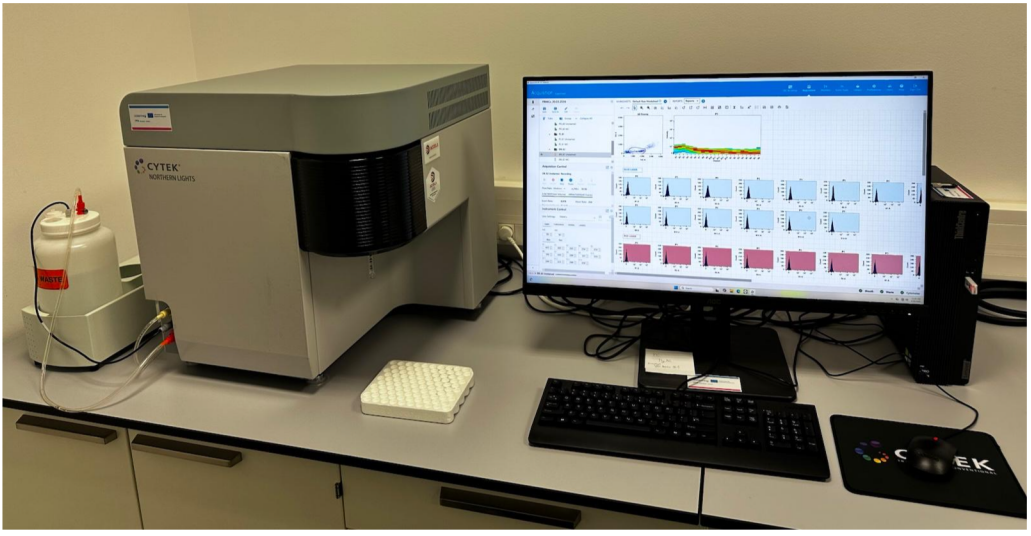

3.

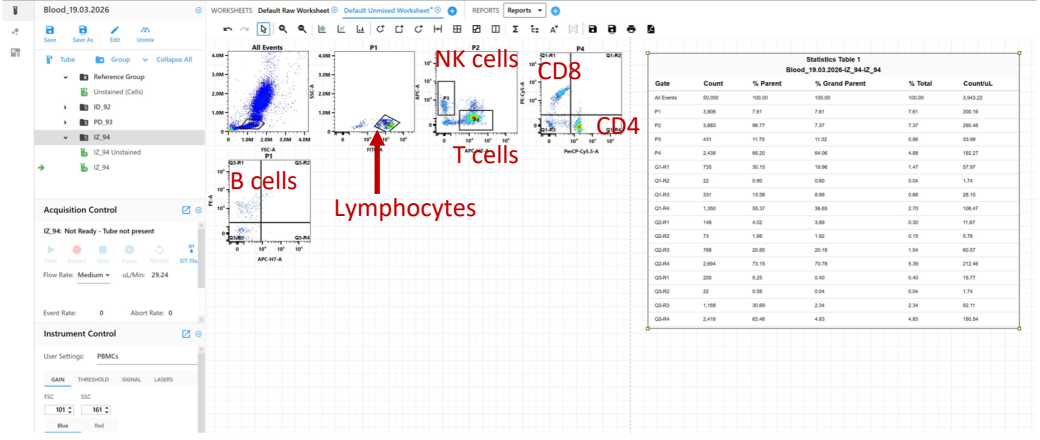

4.


7.

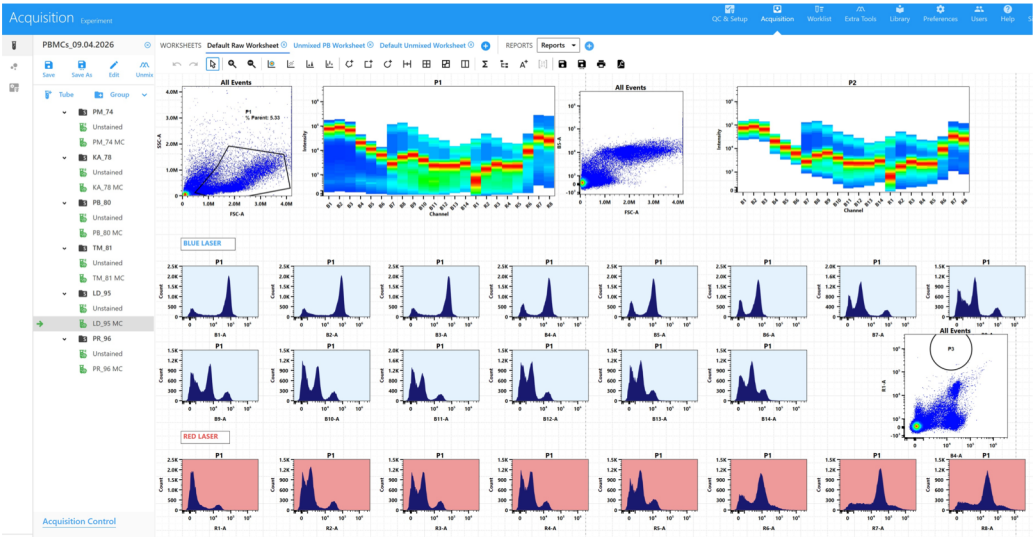
Data acquisition on Cytek Northern Lights Spectral Flowcytometer

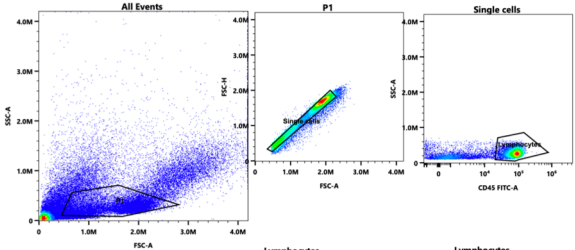
1. Create the Experiment
2. Data acquisition

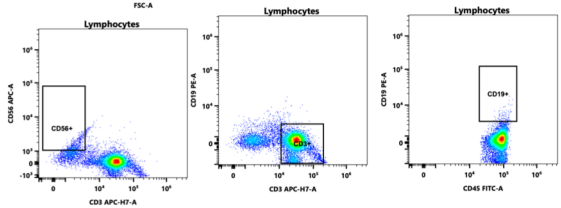


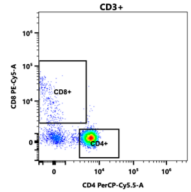


Example of PBMCs immunophenotyping with statistic analysis





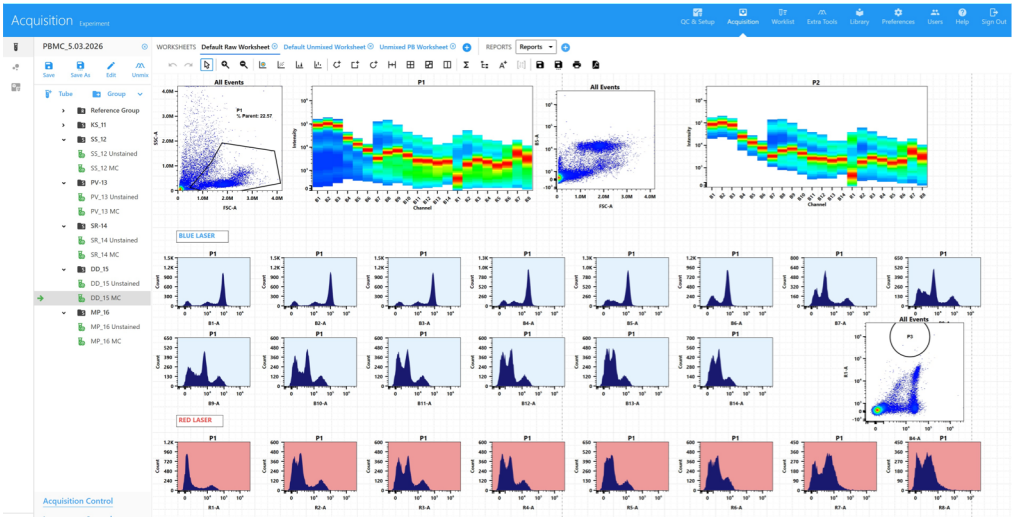


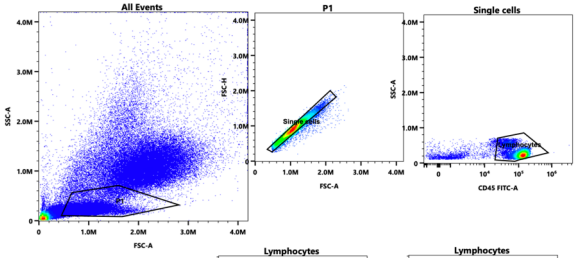


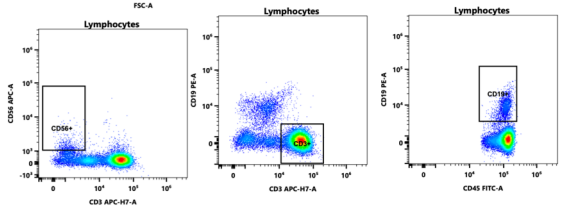
Lymphocyte populations
PBMCs_09.04.2026-LD_95-LD_95 MC

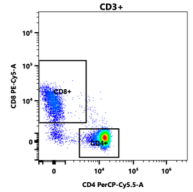
Gate	Count	% Parent	Count/uL
CD56+	479	4.34	8.09
CD3+	6,670	60.39	112.63
CD4+	4,407	66.07	74.42
CD8+	306	4.59	5.17
CD19+	101	0.91	1.71

Example of peripheral blood immunophenotyping with statistic analysis









Lymphocyte populations
PB_31.03.2026-DB-101-Tube_001

Gate	Count	% Parent	Count/uL
CD56+	501	2.89	6.60
CD3+	11,674	67.30	153.83
CD4+	8,307	71.16	109.46
CD8+	2,701	23.14	35.59
CD19+	1,999	11.52	26.34

Working Protocol for Obtaining and Culture Expansion of Tumor Cells

Reagents and consumables	Equipments
PBS	Laminar flow hood
DMEM	Centrifuge
FCS/FBS	Automated pipettes
Antibiotic solution Pen/Strep	Medimachine device
Antibiotic / antifungal solution	Ultrafreezer -80° C
FGF solution	
Tumor tissue – biopsy or tissue fragment	
Pasteur pipettes	
Falcon tubes50 ml	
Tubes 5 ml	
Sterile gloves	
Adherent 24-well plates, sterile	
Sterile scalpel	
Petri dishes ϕ de 3 cm/ 5 cm	
Insulin syringes, detachable needle 1 ml	
Strainers – compatible with 5 ml tubes, pore size 50-100 μm	
Medimachine grinding disposable	

Initially, the culture media for tumor cells (TCs) and tumor-associated fibroblasts (TAFs), as well as the antibiotic and antifungal solution, are prepared.

Antibiotic and antifungal solution

Colistin: vial 80 mg = 1,000,000 IU – dissolve in 10 ml PBS. 9 ml is aliquoted and stored at -80° C. 1 ml of this solution is diluted 1:1 with PBS ⇒ 2 ml of working solution with a concentration of 4 mg/ml (solution A);

Fluconazole: infusion solution, concentration 2 mg/ml;

Metronidazole: infusion solution 5 mg/ml;

Cefazolin: powder vial 1 g; dissolve in 10 ml PBS. 9 ml is aliquoted and stored at -80° C. 1 ml, containing 100 mg cefazolin, constitutes the working solution (solution B).

Prepare the mixture as follows:

- **200 μl** solution A (Colistin)
- **800 μl** Fluconazole solution
- **400 μl** Metronidazole solution
- **200 μl** solution B (Cefazolin)
- **8.4 ml** PBS

The resulting 10 ml mixture is aliquoted into 1 ml tubes and stored at -80° C. **10 µl/ml** of this mixture is added to the culture medium for tumor cells (TCs), the culture medium for peritumoral fibroblasts (TAFs), respectively to the washing solution (PBS). In this way, the following are the useful concentrations achieved:

- Colistin: 4 µg /ml medium
- Fluconazole: 8 µg /ml medium
- Metronidazole: 10 µg /ml medium
- Cefazolin: 100 µg /ml medium

Culture medium for tumor cells (TCs)

DMEM + 10% FCS/FBS + 1% Pen/Strep + 10 µl/ml Antibiotic/Antifungal Solution

Tumor-associated fibroblasts culture medium (TAFs)

DMEM + 10% FCS/FBS + 10 ng/ml FGF + 1% Pen/Strep + 10 µl /ml Antibiotic/Antifungal Solution

Tumor tissue sample - biopsy

1. The fragments obtained by biopsy are of variable size and in variable number, transported in liquid medium (PBS + antibiotic and antifungal solution);
2. The fragments received are counted and a biopsy fragment is selected which will be placed in a 1.5 ml tube with 500 µl of RNA / DNA shield solution (see DNA purification protocol from fresh tumor tissue) being used for DNA purification and subsequent use in the gene sequencing (NGS) protocol; The remaining fragments are divided into 2 approximately equal parts for mechanical dissociation with the Medimachine device;
3. Carefully remove the biopsy fragments using a Pasteur pipette and place in a 3 cm/ 5 cm ϕ Petri dish;
4. Wash with PBS + antibiotic and antifungal solution for 15 minutes;
5. Remove half of the fragments and place in the sterile grinding chamber, adding 1 ml of PBS + antibiotic and antifungal solution (sterile); carefully close the grinding chamber and position in the Medimachine device;
6. Turn on the Medimachine device and leave the samples to grind for 3 minutes, then turn off the device; Carefully remove the grinding chamber and process the contents further in the laminar flow hood;
7. Using a 1 ml syringe (without needle, insulin syringe), remove the liquid fraction of the ground sample and filter it through filters compatible with 5 ml tubes, with a pore size of 50-100 µm;

8. The larger, unground fragments are removed with a Pasteur pipette and placed in 2 wells of a 24-well plate in a fume hood and left to dry;
9. Repeat steps 5 to 8 for the other part of the biopsy sample, using the same filter, but a different 5 ml tube; at the end of this step we will have 2 5 ml tubes containing 1 ml of cell suspension each and 4 wells with solid biopsy samples;
10. Add 1 ml of PBS + antibiotic/antifungal solution to each tube and centrifuge the 2 5 ml tubes with the cell suspension at 1500 rpm for 10 minutes at room temperature, maximum acceleration (9), maximum deceleration (9);
11. Discard the supernatant and resuspend the sediment as follows: 1 tube in 4 ml medium for tumor cells (TCs) and 1 tube in 4 ml medium for peritumoral fibroblasts (TAFs);
12. The cells from the 2 tubes are seeded in the 24-well plate, the content of each tube being sufficient for 4 wells, 1 ml of cell suspension/well;
13. At the end of step 12, carefully add (without removing the solid fragments previously placed in the 4 wells) 1 ml of TAFs medium/well;
14. At the end of the procedure, we will have 12 wells occupied in the 24-well plate as follows: 4 wells with tumor cells (TCs), 4 wells with peritumoral fibroblasts (TAFs) and 4 wells with explant tissue (TAFs_Ex);
15. The culture plate is positioned in the incubator, at 37^{o}C with 5% CO_2 and humidity;
16. The culture plate medium is checked daily to monitor the evolution of the cells and to detect the appearance of infections early (especially in the case of colon tumors); The medium is changed after 7 days from seeding; solid fragments are removed from the culture plate after 4-10 days and the cultivation of TAFs_Ex is continued similarly to that of TAFs obtained by mechanical dissociation.

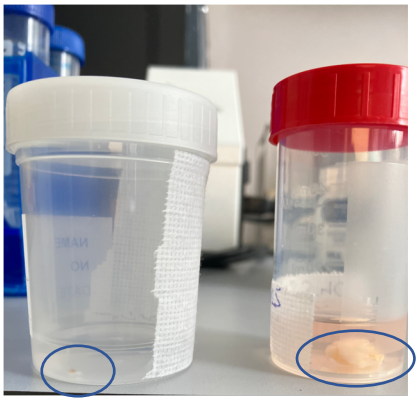
Labeling of culture plates/cryogenic tubes

- All plates (wells) and subsequently cryogenic tubes will have the patient code, formed by the initials of the name and the sample number from the Sample Reception register within the CrossCare project (e.g.: XY_15)
- Wells in the culture plate with TCs will be labeled as follows: patient code + TCs (e.g.: XY_15_TCs)
- Wells in the culture plate with TAFs will be labeled as follows: patient code + TAFs (e.g.: XY_15_TAFs)
- Subsequently, the cryogenic tubes for TCs will be labeled as follows: patient code + TCs + tube no. (e.g.: XY_15_TCs_1 and XY_15_TCs_2 etc.)

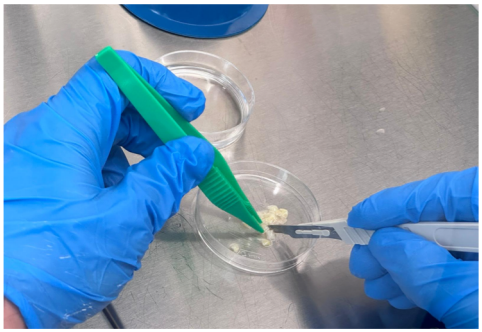
- Subsequently, the cryogenic tubes for TAFs will be labeled as follows: patient code + TAFs + no. tube (e.g.: XY_15_TAFs_1 and XY_15_TAFs_2 etc.)
- All tubes will be marked with the date on which the products obtained from fresh tumor tissue samples were frozen;
- All this information (no. of tubes of each cell type, date of freezing etc.) will also be noted in the CrossCare project register

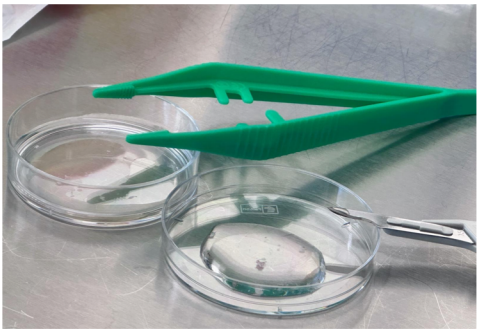
Labeling examples:

XY_15_TCs_1	XY_15_TCs_2	XY_15_TAFs_1	XY_15_TAFs_2
30.03.2025	30.03.2025	01.04.2025	01.04.2025

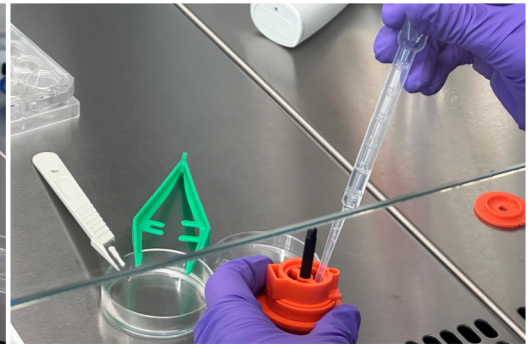


Tumors of variable sizes are processed using a forceps and scalpel in a Petri dish

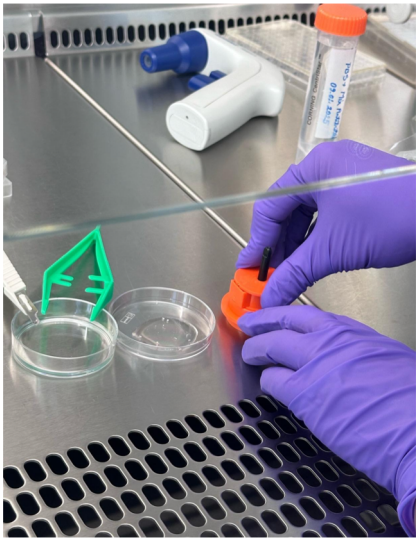




Tumor tissue is cut in small pieces and placed in PBS with antibiotics solution

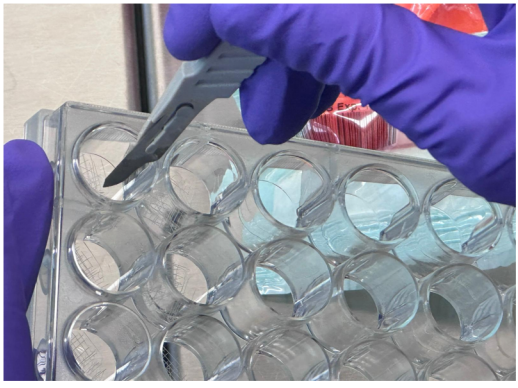


Using a Pasteur pipette, the tumor tissue fragments and 1 ml PBS are transferred to the Medicons grinding chamber. The entire volume of PBS and all tumor tissue fragments are transferred in successive steps, as the previous cellular suspension is removed for further steps from the Medicons chamber.

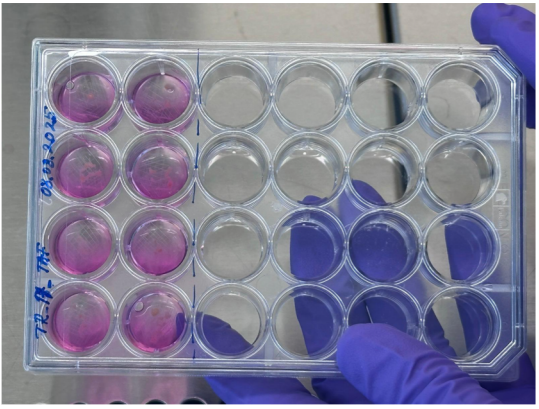


The Medicons grinding chamber is closed and placed in the Medimachine device to grind for 3 minutes. The same procedure is performed for all tumor tissue fragments.

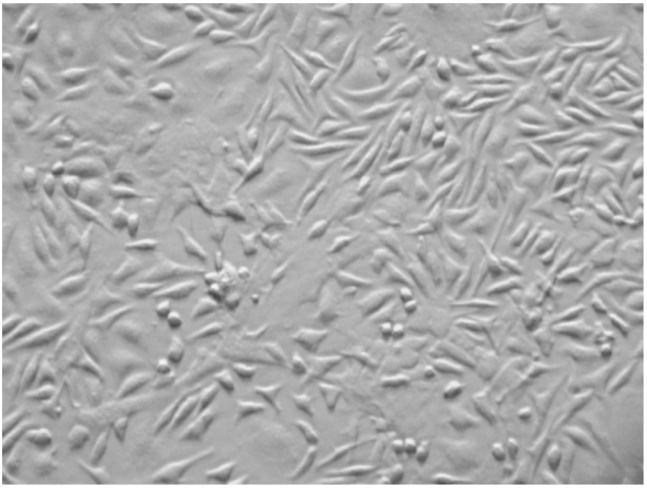
After 3 minutes grinding, the cellular suspension is removed using an insulin syringe with detachable needle and filtered through 100 µm mesh Falcon tube strainer.



For obtaining adherent cells populations using the explant method, the 24-well culture flasks are scratched with a scalpel, so the surface will allow adhesion of cells emerging from the tumor tissue fragments. 2 mm size tissue fragments are placed in the wells and left to adhere on the surface by holding them with half-opened lid, in the laminar flow hood for 30 minutes.

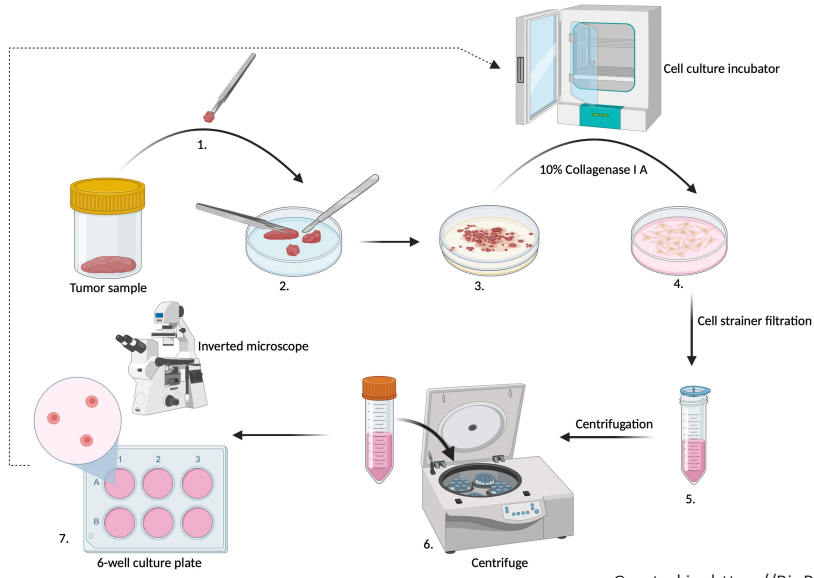


Culture medium containing DMEM, 10% FCS/FBS, 10 ng/ml FGF and 1% Pen/Strep solution is added on the tissue fragments and the culture plate is placed into the cell culture incubator, at 37°C with 5% CO_2 and humidity.



Morphological aspects of adherent cells, fibroblast shape, in culture plates, under direct light microscope.

Working Protocol for Obtaining and Culture Expansion of Tumor Cells (TCs) and Tumor-Associated Fibroblasts (TAFs)



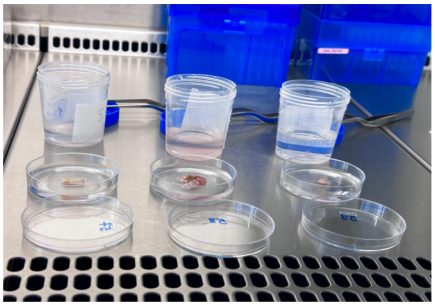
Created in https://BioRender.com

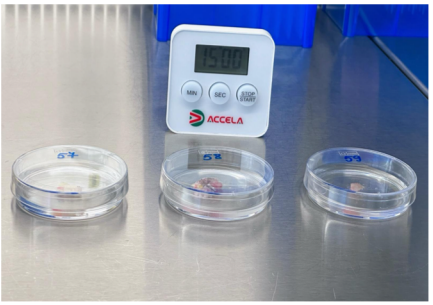
Reagents and consumables	Equipments
PBS + antibiotics solution	Laminar flow hood
DMEM (Dulbecco's Modified Eagle Medium)	Centrifuge
DMSO	Micopipettes
FGF (Fibroblast Growth Factor)	Cryobox
FCS/FBS (Fetal Calf/Bovine Serum)	Ultrafreezer -80° C
Antibiotic solution Pen/Strep	Inverted light microscope
Collagenase I A (from Clostridium histolyticum)	Cell culture incubator (5% CO_2)
Petri dishes (ø 35, 50 mm)	
Cell strainers 40 µm	
Cryogenic vials (2 ml)	
Sterile gloves	
Pasteur pipettes	
Falcon tubes 50 ml	
Scissors	
Forceps	

1. Carefully remove the tumor tissue fragments from the transport recipient using a Pasteur pipette;
2. Place the tissue fragments in a 5 cm ϕ Petri dish containing PBS + antibiotics/antifungal mix and wash them for 15 minutes, in the laminar flow hood, with closed lid;
3. Transfer the washed tumor tissue after 15 minutes in another clean 3 cm ϕ Petri dish;
4. Cut part of the tumor tissue (approximatively 0.5-1 cm^3) in smaller pieces, one by one using forceps and scissors, in the laminar flow hood;
5. Add 2 ml of tumor cells medium in the 3 cm ø Petri dish, for each tumor fragment: DMEM + 10% FCS + 1% P/S;
6. Add 10% volume of Collagenase IA (200 µl) for each tumor fragment;
7. Place the Petri dishes in a cell culture incubator, at 37°C, 5% CO_2 and humidity for at least 30 minutes, or until the tissue appears dissociated by the enzyme;
8. Prepare for obtaining unicellular suspension from the enzymatically dissociated tumor tissue;
9. Take the Petri dishes out of the incubator and transfer the medium and dissociated tissue to a 50 ml Falcon tube
10. Use a 70 µm mesh size cell strainer, placed on top of the Falcon tube, for filtration of the cellular suspension, so that the larger fragments, undissociated will be removed;
11. Add 5 ml of PBS in the Falcon tube containing the filtered dissociated tumor tissue and wash by centrifugation at 1500 rpm, for 10 min. at room temperature
12. Discard the supernatant and add 10 ml of culture medium (DMEM + 10% FCS + 1% P/S) in the same 50 ml Falcon tube for the second wash;
13. wash by centrifugation at 1500 rpm, for 10 min. at room temperature
14. After filtration and 2 steps washing, resuspend the cellular pellet in 1 ml of culture medium and count the cells using a hemacytometer (Trypan Blue viable dye, 1:1 ratio);
15. Further divide the suspension cells in 2: one part for cryopreservation, another part for cell culture;
16. Cryopreserve up to 2 x 10^6 cells are preserved in 2 ml cryogenic vials in 10% DMSO for further use;
17. Place in 6-well culture plates up to 1 x 10^6 cells and cultivated the cells in culture medium containing DMEM + 10%FCS + 1% P/S + 10 ng/ml FGF (3 ml/well);

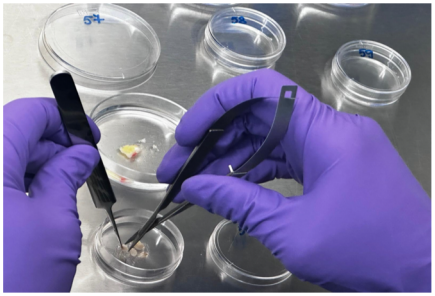
18. Put the culture plates in the cell incubator (37°C, 5% CO_2 and humidity) for long term cell culture of TAFs;
19. Check the culture plate daily to monitor the evolution of the cells and to detect the appearance of infections early (especially in the case of colon tumors);
20. Replace the medium every 3-4 days with fresh culture medium.

Samples, consumables and reagents needed for the protocol

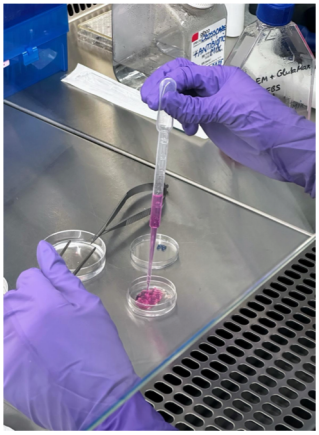




Tissue fragments were placed in a 5 cm ϕ Petri dish containing PBS + antibiotics/antifungal mix for 15 minutes, in the laminar flow hood, with closed lid;



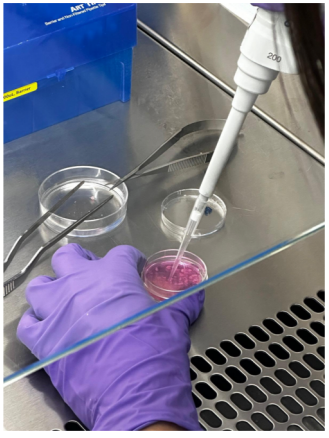
Part of the tumor tissue (approximatively 0.5-1 cm^3) cut in small pieces, one by one using forceps and scissors, in the laminar flow hood;



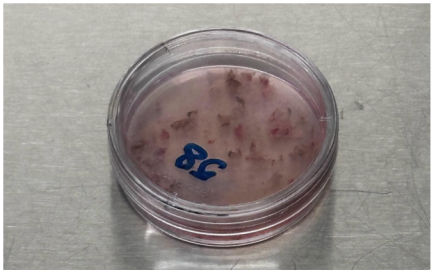
Addition of 2 ml of cell culture medium in the 3 cm ϕ Petri dish: DMEM + 10% FCS + 1% P/S;

Collagenase from Clostridium histolyticum type IA was resuspended in PBS + calcium chloride and allicoted at 10 mg/ml concentration

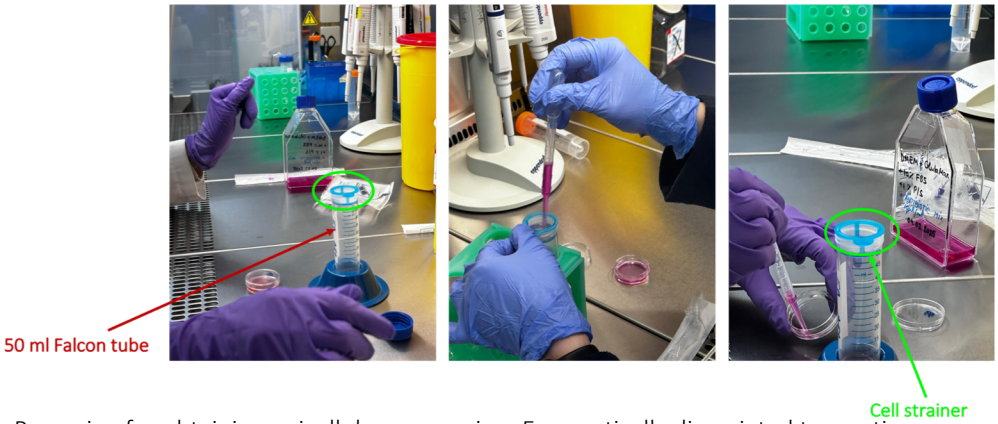
Allicoted Collagenase type IA



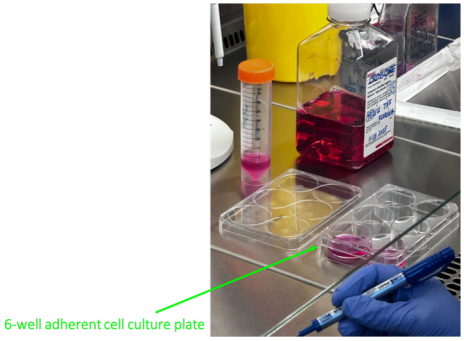
10% volume of Collagenase IA 10 mg/ml (200 µl) was added for each tumor; further, the Petri dishes containing the mixture were placed in a cell culture incubator, at 37°C, 5% CO_2 and humidity for at least 30 minutes, or until the tissue appears dissociated by the enzyme;



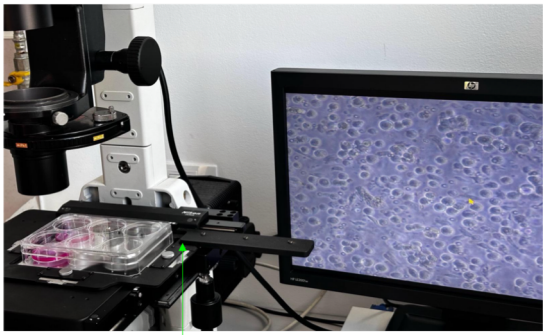
Macroscopic aspect of the Petri dish containing the dissociated tumor tissue after 30 minutes incubation



Preparing for obtaining unicellular suspension. Enzymatically dissociated tumor tissue transferred for filtration - cell strainers of 40 µm mesh size



The filtered fraction washed by centrifugation at 1500 rpm, for 10 min. at rt in a 50 ml Falcon tube and then resuspended in culture medium. The culture plates are placed in the cell culture incubator (37°C, 5% CO_2 and humidity) for long term cell culture



Upright microscope;
inverted light microscope

Macroscopic and microscopic view of the cell culture plate and cells

Working Protocol for Cytotoxic Assays

Objective of the protocol: The cell number, drugs, doses and analysis methods will be detailed within this protocol, so that the assay could be reproducible. The cultivated cells, with identified genomic alterations and therapeutic indications will be tested in cytotoxic assays, based on spectrophotometric evaluation, according to manufacturer protocols.

Summary plate design (96-well, triplicates)

Each condition in triplicate (3 wells). Layout (rows A-H, columns 1-12) - simple example:

- Columns 1-2: Vehicle control (cells + vehicle) - 6 wells total (triplicate A & duplicate B)
- Columns 3-4: Cell-only (untreated) control (baseline viability)
- Column 5: Max-lysis control (cells + lysis buffer / 0.1–1% Triton X-100) - for CellTox and CellTiter-Glo normalization
- Column 6: Medium blank (no cells) - background (with reagent)
- Columns 7-12: Substances A-F (each substance occupies one column, in triplicate wells across two rows to make 3 wells per substance)

You can also reserve extra columns for positive control compound (known cytotoxin) and dose-response series. Below I assume single test concentration per substance; if you want dose-response use 3-7 concentrations and reduce replication or use extra plates.

Reagents & consumables

- CellTox™ Green (Promega) or equivalent membrane-impermeant DNA dye (follow vendor for storage/handling). White, opaque, tissue-culture-treated 96-well plates (for luminescence: use white plates - better signal).
- CellTiter-Glo® Luminescent Cell Viability Assay (Promega) or equivalent ATP-based luminescence kit. Black, clear-bottom 96-well plates (for fluorescence reads like CellTox Green, adherent cells).

Note: you may run both assays in the same plate only if plate choice and optical needs are compatible (common strategy: use black plates for CellTox Green kinetics, then transfer aliquot to white plate or run CellTiter-Glo in same plate if manufacturer allows). Running CellTox Green and CellTiter-Glo on separate plates is recommended to avoid cross-optical compromises unless you validate compatibility.

- Phenol-red-free medium for fluorescence assays if possible.
- Positive control cytotoxicant (e.g., staurosporine or 1% Triton X-100 for max lysis), vehicle solvent (DMSO, ≤0.5% final), PBS.
- Plate seal (breathable) for incubations if needed.
- Multichannel pipettes, reservoirs, sterile tips.
- VarioScan LUX plate reader (set to appropriate filters/wavelengths; see settings section).

Seeding (typical 96-well)

Adherent cells: seed to reach ~70-80% confluence at read time. Typical starting densities (cells/well, 100 µL):

- Low-growth epithelial line (e.g., MCF7): 5,000-10,000 cells/well
- Fast-growing lines (e.g., HEK293, HeLa): 8,000-15,000 cells/well

Suspension cells: seed at higher starting counts to ensure measurable signal:

- 20,000-50,000 cells/well (depends on cell type and assay sensitivity)
- Seed cells in 100 µL medium and allow attachment for adherent cells 16-24 h (overnight) before treatment. For suspension lines, allow 1-2 h equilibration before adding compounds.

Treatment

- Add substances A-F at desired concentration(s) in 100 µL total volume (final DMSO ≤0.5%). Keep vehicle control with same vehicle concentration.
- Incubation times: choose based on mechanism - recommended timepoints for CellTox kinetics: 0 h (immediately after addition), 2 h, 6 h, 24 h, 48 h. For CellTiter-Glo endpoint choose 24 or 48 h depending on expected effect.
- For immune-mediated killing or slow cytotoxicity extend to 72 h with medium refresh as needed.

CellTox™ Green - real-time cytotoxicity (general workflow)

CellTox Green is add-and-read: dye binds DNA of cells with compromised membranes → fluorescent signal ∝ dead cells.

- Plate type: black, clear-bottom plate for adherent cells (bottom read) or black plate for suspension (top read recommended after gentle spin; see below). Use phenol-red-free medium.
- Prepare CellTox Green reagent: dilute as manufacturer instructs to make a 2X or 1X working solution if required. (Follow kit insert.)

- Add dye: For an add-and-read format, add dye to wells to reach final 1X concentration (many protocols add 10% v/v of 10x stock or a specified µL per well). Follow kit recommended volumes.

Practical example: if starting volume is 100 µL/well and the kit requires 1X final, add 25 µL of 4x reagent to reach 125 µL final (this is illustrative - verify with kit).

- Incubate briefly (5-10 min at RT) to homogenize then begin reads. For adherent cells you can read immediately.
- Reading schedule (kinetics): read plates at desired timepoints (0, 2, 6, 24, 48 h). Keep plates at 37 °C between reads (use incubator or plate reader incubation if available). Minimize light exposure between reads.
- For suspension cells: to improve signal and reduce background, centrifuge gently just before reading (300 × g for 3 min) to settle cells to bottom, then read from top or bottom depending on reader geometry. Alternatively, transfer cells to a clear-bottom plate and settle prior to reading.

Instrument settings for fluorescence (VarioScan LUX):

- Excitation: ~485 nm (bandwidth ~20 nm)
- Emission: ~520 nm (bandwidth ~20 nm)
- Read mode: bottom read for adherent; top read or bottom after centrifugation for suspension (optimize).
- Gain: automatic or set to place vehicle control mid-range.
- Integration time: 50–200 ms (optimize to avoid saturation).

Controls: medium blank (no cells), vehicle control (cells+vehicle), cell-only control (untreated), max-lysis (cells + lysis agent; defines 100% dead) - measure these at same timepoints.

Data analysis: compute % cytotoxicity at each timepoint:

$$\% \text{ Cytotoxicity} = 100 * (RFU\_sample - RFU\_lowControl) / (RFU\_maxLysis - RFU\_lowControl)$$

Where:
RFU_sample = fluorescence of treated well
RFU_lowControl = fluorescence of vehicle control (baseline dead cells)
RFU_maxLysis = fluorescence of max-lysis control (e.g., Triton X-100)
If background (medium blank) contributes, subtract blank RFU from all wells first.

CellTiter-Glo® - endpoint ATP viability (general workflow)

CellTiter-Glo lyses cells and generates luminescence proportional to ATP (viable cells).

- Plate type: white opaque 96-well plates for best signal (some kits allow clear plates but signal lower). If you ran CellTox Green in same plate, validate compatibility; otherwise use a separate plate for CellTiter-Glo.
- Bring CellTiter-Glo reagent to room temperature and equilibrate per kit instructions. Prepare any required dilutions.
- At endpoint (e.g., 24 or 48 h): equilibrate plate to room temperature for ~20 min if recommended (some labs read directly).
- Add reagent: typical recommended ratio is 1:1 reagent:well volume (e.g., add 100 µL CellTiter-Glo to 100 µL medium). Mix gently for 2 min on an orbital shaker to induce cell lysis and maximize signal.
- Incubation: allow luminescent signal to stabilize (typically 10 min at RT; some protocols allow 10–30 min).

Instrument settings for luminescence (VarioScan LUX):

- Read mode: luminescence, top read (or default; VarioScan allows top/bottom)
- Integration time: 0.5–1.0 s per well (start 0.5 s, increase if signal low)
- Settling time: 100–300 ms
- Gain: auto or adjust to avoid saturation

Controls: medium blank (no cells + reagent), vehicle control (cells + vehicle), cell-only control (untreated), max-lysis control (treat with 0.1-1% Triton X-100 15-30 min before reagent).

Data analysis: compute % viability (relative to vehicle):

$$\text{Relative viability (\%)} = 100 * (\text{RLU\_sample} - \text{RLU\_blank}) / (\text{RLU\_vehicle} - \text{RLU\_blank})$$

Where:
RLU_sample = luminescence of treated well
RLU_blank = luminescence of medium blank with reagent
RLU_vehicle = luminescence of vehicle control (cells + vehicle)
You can convert to % cytotoxicity = 100 - Relative viability.

Combined workflow suggestions (both assays)

Option A (separate plates): Run CellTox Green kinetics on one plate (black), then in a parallel plate perform CellTiter-Glo at the chosen endpoint (white). This avoids compatibility issues and gives both real-time death kinetics and endpoint viability.

Option B (single plate, validated): If you must use the same wells, run CellTox Green (non-lytic) throughout, then at endpoint add CellTiter-Glo to the same wells and read luminescence — but validate that the CellTox reagent does not quench/alter ATP signal. Many labs do this, but only after pilot validation.

Protocol for DNA Purification Using PureLink™ Genomic DNA Kit

1. Set a water bath or heat block at 55°C.
2. Add 20 µl Proteinase K to a sterile microcentrifuge tube.
3. Process cells from tumor samples: up to 5 x 10^6 cells, harvest cells by centrifugation. Remove the growth medium. Resuspend cells in 200 µl PBS.
4. Transfer 200 µl cells or blood in PBS to the tube containing Proteinase K from Step 2.
5. Add 20 µl RNase A to the sample. Mix well by brief vortexing and incubate at room temperature for 2 minutes.
6. Add 200 µl PureLink™ Genomic Lysis/Binding Buffer and mix well by vortexing to obtain a homogenous solution.
7. Incubate at 55°C for 10 minutes to promote protein digestion.
8. Add 200 µl 96-100% ethanol to the lysate. Mix well by vortexing to yield a homogenous solution.
9. Proceed immediately to **Purification Protocol**

The purification procedure is designed for purifying genomic DNA using a spin column-based centrifugation procedure in a total time of **10-15 minutes**.

1. Remove a PureLink™ Spin Column in a Collection Tube from the package.
2. Add the lysate (~640 µl) prepared with PureLink™ Genomic Lysis/Binding Buffer and ethanol to the spin column.
3. Centrifuge the column at 10,000 × g for 1 minute at room temperature.
4. Discard the collection tube and place the spin column into a clean PureLink™ Collection Tube supplied with the kit.
5. Add 500 µl Wash Buffer 1 prepared with ethanol to the column.
6. Centrifuge column at 10,000 × g for 1 minute at room temperature.
7. Discard the collection tube and place the spin column into a clean PureLink™ collection tube supplied with the kit.
8. Add 500 µl Wash Buffer 2 prepared with ethanol to the column.
9. Centrifuge the column at maximum speed for 3 minutes at room temperature. Discard collection tube.
10. Place the spin column in a sterile 1.5-ml microcentrifuge tube.

11. Add 25-200 µl of PureLink™ Genomic Elution Buffer to the column. Choose the suitable elution volume for your needs.
12. Incubate at room temperature for 1 minute. Centrifuge the column at maximum speed for 1 minute at room temperature.

The tube contains purified genomic DNA.

13. To recover more DNA, perform a second elution step using the same elution buffer volume as first elution.
14. Centrifuge the column at maximum speed for 1.5 minutes at room temperature.

The tube contains purified DNA. Remove and discard the column.

15. Use DNA for the desired downstream application or store the purified DNA at 4°C (short-term) or -20 °C (long-term).

Working Protocol for DNA Purification Using PureLink™ Genomic DNA Kit

Tissue lysate protocol

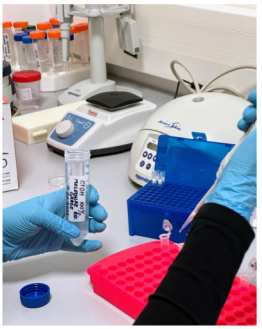
Reagents and consumables	Equipments
Tumor tissue stored in DNA/RNA shield	Thermoblock (55° C)
Pure Link Genomic DNA Kit	Minicentrifuge
1.5 DN-ase / RN-ase – free tubes	Micropipettes
Pasteur pipettes	Vortex
Scalpel	Freezer -80° C
Sterile gloves	

1. Set a water bath or heat block at 55°C.
2. Place up to 25 mg of minced mammalian tissue into a sterile microcentrifuge tube
3. Add 180 µl PureLinkTM Genomic Digestion Buffer and 20 µl Proteinase K to the tube. Ensure the tissue is completely immersed in the buffer mix.

4. Incubate at 55°C with occasional vortexing until lysis is complete (1-4 hours). For larger tissue pieces, you may perform overnight digestion.
5. To remove any particulate materials, centrifuge the lysate at maximum speed for 3 minutes at room temperature. Transfer supernatant to a new, sterile microcentrifuge tube.

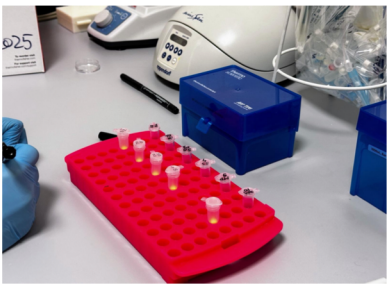
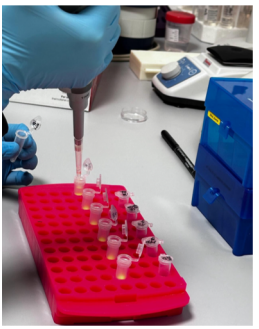
6. Add 20 µl RNase A to lysate, mix well by brief vortexing, and incubate at room temperature for 2 minutes.
7. Add 200 µl PureLinkTM Genomic Lysis/Binding Buffer and mix well by vortexing to yield a homogenous solution.
8. Add 200 µl 96-100% ethanol to the lysate. Mix well by vortexing to yield a homogenous solution.
9. Proceed immediately to **Purification Protocol**



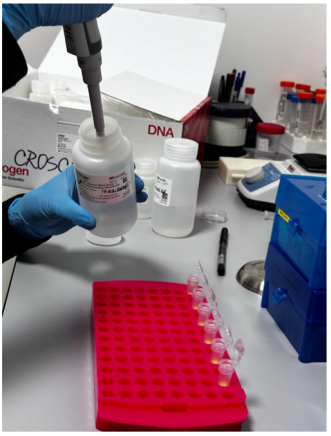


DNA purification protocol

1. Remove a PureLinkTM Spin Column in a Collection Tube from the package.
2. Add the lysate (~640 µl) prepared with PureLinkTM Genomic Lysis/Binding Buffer and ethanol to the spin column.
3. Centrifuge the column at 10,000 × g for 1 minute at room temperature

4. Discard the collection tube and place the spin column into a clean PureLinkTM Collection Tube supplied with the kit.
5. Add 500 µl Wash Buffer 1 prepared with ethanol (page 2) to the column.
6. Centrifuge column at 10,000 × g for 1 minute at room temperature.
7. Discard the collection tube and place the spin column into a clean PureLinkTM collection tube supplied with the kit.
8. Add 500 µl Wash Buffer 2 prepared with ethanol (page 2) to the column.
9. Centrifuge the column at maximum speed for 3 minutes at room temperature. Discard collection tube.
10. Place the spin column in a sterile 1.5-ml microcentrifuge tube



11. Add 25-200 µl of PureLinkTM Genomic Elution Buffer to the column. Choose the suitable elution volume for your needs (we used volumes of 75 and 50 µl).
12. Incubate at room temperature for 1 minute. Centrifuge the column at maximum speed for 1 minute at room temperature.
13. *The tube contains purified genomic DNA.*
14. To recover more DNA, perform a second elution step using the same elution buffer volume as first elution.
15. Centrifuge the column at maximum speed for 1.5 minutes at room temperature.
16. *The tube contains purified DNA.* Remove and discard the column.
17. Use DNA for the desired downstream application or store the purified DNA at 4oC (short-term) or -20°C (long-term).

Protocol for Qubit dsDNA HS Assay Kit

The Qubit dsDNA HS (High Sensitivity) Assay Kits make DNA quantitation easy and accurate. The assay is highly selective for double- stranded DNA (dsDNA) over RNA. Depending on sample volume, the assay is accurate for initial sample concentrations from 5 pg/µL to 120 ng/µL providing an assay range of 0.1-120 ng. Common contaminants such as salts, free nucleotides, solvents, detergents, or protein are well tolerated in the assay. This Qubit assay kit can be used with any Qubit Fluorometer.

Critical assay parameters

Assay temperature

Qubit assays deliver optimal performance when all solutions are at room temperature; temperature fluctuations can influence the accuracy of the assay.

To minimize temperature fluctuations, insert all assay tubes into the Qubit Fluorometer only for as much time as it takes for the instrument to measure the fluorescence. Qubit Fluorometers can raise the temperature of the assay solution significantly, even over a period of a few minutes. Do not hold the assay tubes in your hand before reading because this warms the solution and results in a different reading.

Incubation time

To allow the Qubit assay to reach optimal fluorescence, incubate the tubes for the DNA and RNA assays for 2 minutes after mixing the sample or standard with the working solution. After this incubation period, the fluorescence signal is stable for 3 hours at room temperature when the samples and standards are protected from light.

Calibrate the Qubit Fluorometer

For each assay, you have the option to run a new calibration or use values from the previous calibration. To minimize variables that can affect performance, performing a new calibration for every new assay run is strongly recommended. See Figure 1 for an example of the calibration curve used to generate the quantification results.

Photostability of the Qubit reagents

The Qubit reagents exhibit high photostability in the Qubit Fluorometer, showing <0.3% drop in fluorescence after 9 readings and <2.5% drop in fluorescence after 40 readings. However, if the assay tube remains in the Qubit Fluorometer for multiple readings, a temporary reduction in fluorescence will be observed as the solution increases in temperature. Note that the temperature inside the QubitFluorometer may be as much as 3°C above room temperature after 1 hour. For this reason, if you want to perform multiple readings of a single tube, remove the tube from the instrument and let it equilibrate to room temperature for 30 seconds before taking another reading.

Prepare samples and standards

1. Set up the required number of Qubit tubes for standards and samples. The Qubit dsDNA HS Assay requires 2 standards.

Note: Use only thin-wall, clear, 0.5-mL PCR tubes for the Qubit 4 Fluorometer

2. Label the tube lids.

Note: Do not label the side of the tube as this could interfere with the sample read. Label the lid of each standard tube correctly. Calibration of the Qubit Fluorometer requires the standards to be inserted into the instrument in the right order.

3. Prepare the Qubit working solution by diluting the Qubit dsDNA HS Reagent 1:200 in Qubit dsDNA HS Buffer. Use a clean plastic tube each time you prepare the Qubit working solution.

IMPORTANT! Do not mix the working solution in a glass container.

4. Add the Qubit working solution to each tube such that the final volume is 200 µL.

	Standard assay tubes	User sample assay tubes
Volume of working solution	190 µl	180-199 µl
Volume of standard	10 µl	-
Volume of user sample	-	1-20 µl
Total volume in each assay tube	200 µl	200 µl

Note: The final volume in each tube must be 200 µL. Each standard tube requires 190 µL of Qubit working solution, and each sample tube requires anywhere from 180-199 µL. Prepare sufficient Qubit working solution to accommodate all standards and samples. Qubit Fluorometers provide a reagent calculator, which quickly computes the necessary volume of working solution needed.

5. Add 10 µL of each Qubit standard to the appropriate tube.
6. Add 1-20 µL of each user sample to the appropriate tube.

Note: If you are adding 1-2 µL of sample, use a 2-µL pipette for best results.

7. Vigorously vortex for 3-5 seconds. Be careful not to create bubbles.
8. Allow all tubes to incubate at room temperature for 2 minutes, then proceed to read standards and samples (next section).

Read standards and samples

Read samples and standards with the Qubit 3 Fluorometer

1. On the Home screen, touch dsDNA, then select dsDNA High Sensitivity as the assay type. Touch Read standards to proceed.

Note: If you have already performed a calibration for the selected assay, the instrument prompts you to choose between reading new standards and running samples using the previous calibration. If you want to use the previous calibration, skip to step 4. Otherwise, continue with step 2.

2. Insert the tube containing Standard #1 into the sample chamber, close the lid, then touch Read standard. When the reading is complete (~3 seconds), remove Standard #1.
3. Insert the tube containing Standard #2 into the sample chamber, close the lid, then touch Read standard. When the reading is complete, remove Standard #2.

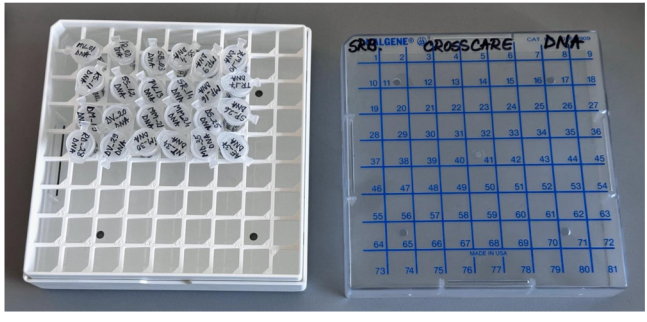
Note: The instrument displays the results on the Read Standards screen. Touch Run samples.

4. On the assay screen, select the Sample volume and units.
   - Touch the + or – buttons on the wheel, or anywhere on the wheel itself, to select the sample volume added to the assay tube (1-20 µL).
   - From the Unit dropdown menu, select the units for the output sample concentration.
5. Insert a sample tube into the sample chamber, close the lid, then touch Read tube. When the reading is complete (~3 seconds), remove the sample tube. The top value (in large font) is the concentration of the original sample and the bottom value is the dilution concentration.
6. Repeat step 6 until all samples have been read.

Working Protocol for DNA Quantification

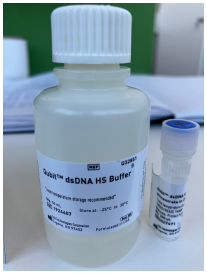
Qubit dsDNA HS Assay Kit - Qubit 3 Fluorometer

Reagents and consumables	Equipments
Sample DNA	Qubit 3 fluorometer
Qubit dsDNA HS Assay kit	Minicentrifuge
1.5 ml DN-ase / RN-ase – free tubes	Micropipettes
0.5 ml Qubit tubes	Vortex
Sterile gloves	



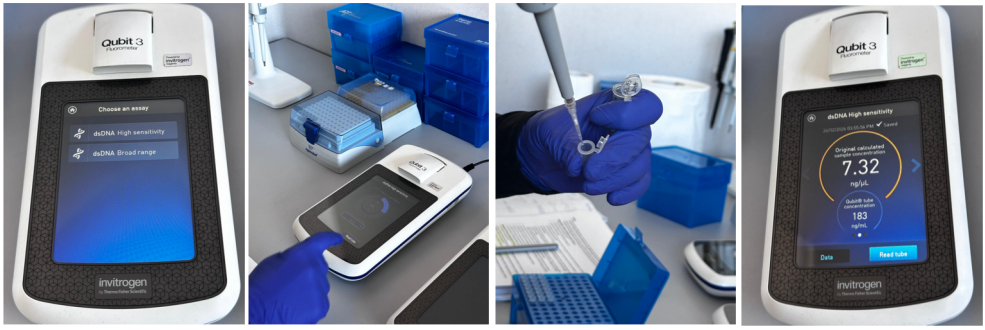
DNA quantification

1. Set up the required number of Qubit tubes for standards and samples. The Qubit dsDNA HS Assay requires 2 standards.
2. Label the tube lids.
3. Prepare the Qubit working solution by diluting the Qubit dsDNA HS Reagent 1:200 in Qubit dsDNA HS Buffer. Use a clean plastic tube each time you prepare the Qubit working solution.
4. Add the Qubit working solution to each tube such that the final volume is 200 µL.

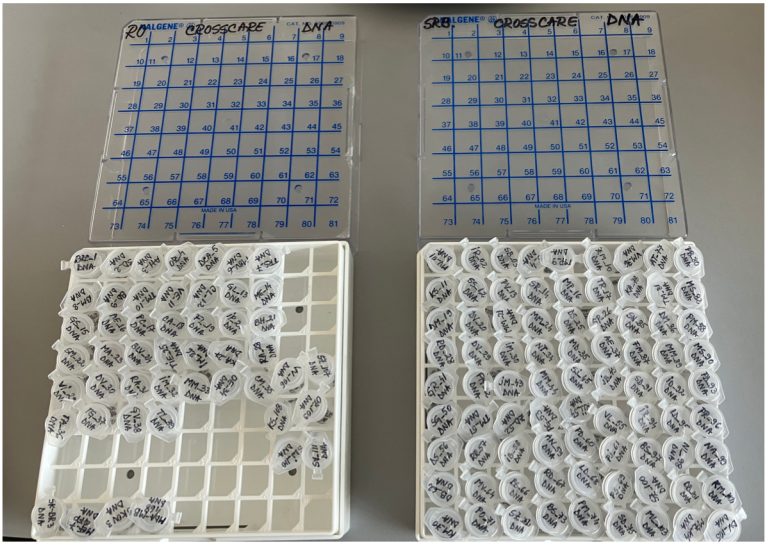


5. Add 10 µL of each Qubit standard to the appropriate tube.
6. Add 1-20 µL of each user sample to the appropriate tube.
7. Vigorously vortex for 3-5 seconds. Be careful not to create bubbles.
8. Allow all tubes to incubate at room temperature for 2 minutes, then proceed to read standards and samples

9. On the Home screen, touch dsDNA, then select dsDNA High Sensitivity as the assay type. Touch Read standards to proceed.
10. On the assay screen, select the Sample volume and units.
    - Touch the + or – buttons on the wheel, or anywhere on the wheel itself, to select the sample volume added to the assay tube (1-20 µL) – we added **5 µL**
    - From the Unit dropdown menu, select the units for the output sample concentration.



11. Insert a sample tube into the sample chamber, close the lid, then touch Read tube. When the reading is complete (~3 seconds), remove the sample tube.
12. **The top value (in large font)** is the concentration of the original sample and **the bottom value** is the dilution concentration.
13. Repeat step 6 until all samples have been read.
14. For DNA sequencing: **15 ng of DNA**



Bench-Side Working Protocol for NGS

Ion AmpliSeq™ Library Preparation and Templating on the Ion Chef™ System

1. Purpose

This protocol describes the preparation of targeted DNA libraries using the Ion Chef™ System with the Ion AmpliSeq™ Kit for Chef DL8 and the Ion AmpliSeq™ Cancer HotSpot Panel v2, followed by preparation for templating and downstream sequencing on Ion Torrent™ platforms. The Ion AmpliSeq™ Cancer HotSpot Panel v2 targets hotspot regions from 50 cancer-associated genes and is optimized for somatic mutation analysis in oncology research.

The protocol is intended for research use and summarizes the practical workflow for:

- genomic DNA preparation,
- automated library construction,
- barcode incorporation,
- library recovery,
- templating preparation,
- quality control steps.

2. Materials and Equipment

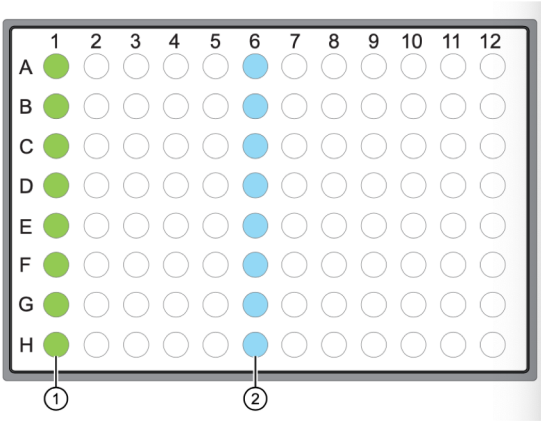
Reagents and consumables	Equipments
Ion AmpliSeq™ Kit for Chef DL8	Ion Chef™ Instrument
Ion AmpliSeq™ Cancer HotSpot Panel v2	Ion Torrent™ sequencing platform
IonCode™ Barcode Adapters	Qubit™ Fluorometer
Ion AmpliSeq™ Chef Solutions DL8 cartridge	Vortex
Ion AmpliSeq™ Chef Reagents DL8 cartridge	Minicentrifuge
Enrichment Cartridge	Calibrated pipettes
IonCode™ 96 Well PCR Plate	
Nuclease-free water	

3. Sample preparation and initial QC

- Extract genomic DNA using validated extraction procedures.
- Quantify DNA using Qubit™ fluorometric quantification.
- Assess DNA purity and integrity.
- Normalize DNA concentration according to panel requirements.
- Transfer samples into the IonCode™ 96 Well PCR Plate.

IMPORTANT:

- Avoid repeated freeze-thaw cycles.
- Use filtered pipette tips.
- Maintain pre-PCR and post-PCR area separation.



Each **column 1 well** contains 15 µL of diluted gDNA sample (0.67 ng/µL, 10 ng total), Direct FFPE DNA, or Nuclease-free Water as non-template control.

Each **column 6 well** contains a dried-down IonCodeTM barcode. The lowest barcode number is in A6, and the highest barcode number is in H6. All appear light blue in the actual plates. Variation in the blue color is normal and does not affect performance or indicate any quality issue with the product.

1. Remove, then discard the plate seal from an IonCodeTM 96-well PCR Plate.
2. Pipet 15 µL of each gDNA sample (0.67 ng/µL, 10 ng), or Direct FFPE DNA sample, into wells A1 to H1 of the plate.

Note:

- If you are processing fewer than 8 samples, it is preferable to add replicates or positive control samples to the run. Otherwise, pipet 15 µL of Nuclease-free Water as non-template control into column 1 wells that do not contain a DNA sample.
- If processing 5 or fewer samples, we recommend that you quantify the output combined library by qPCR to ensure that an optimal concentration is used in templating reactions.

3. Carefully inspect each well for air bubbles. Remove any air bubbles by gentle pipetting. Alternatively, seal the plate with MicroAmpTM Clear Adhesive Film, then centrifuge the plate briefly in a plate centrifuge. Carefully remove the plate seal.

IMPORTANT

- If you are sealing the plate, offset the film to the left so that the adhesive does not cover the barcode label. If the barcode label becomes damaged, you can override the error during the Deck Scan.
- If using the Ion AmpliSeqTM Direct FFPE DNA Kit, start the Ion ChefTM run within 10 minutes of transferring the last sample slurry to the IonCodeTM 96-well PCR plate.
- If ≥10 minutes has elapsed, pipet each sample slurry up and down at least 5 times to mix, load the IonCodeTM 96-well PCR plate onto the Ion ChefTM Instrument, then start the run.

4. Sample set creation in TORRENT SUITE™

- Open Torrent Suite™ Software.
- Create a new Sample Set manually or import using CSV format.
- Select:
  Library Prep Type: “AmpliSeq on Chef”
  Library Prep Kit: “Ion AmpliSeq Kit for Chef DL8”
- Assign:
  sample names,
  barcode IDs,
  plate positions.
- Save the Sample Set and verify correct sample mapping.

The Sample Set information is automatically transferred to the Ion Chef™ workflow.

5. Preparation of primer pools

The Ion AmpliSeq™ Cancer HotSpot Panel v2 primer pools are prepared according to manufacturer recommendations.
For 2X primer pools:

- Add 150 µL Primer Pool into Position A.
- Add 150 µL Primer Pool into Position B.

Mix thoroughly by vortexing and centrifuge briefly before loading.

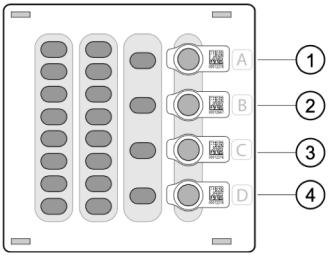
6. Loading the Ion Chef™ instrument

Load the following consumables into the IonChef Instrument:

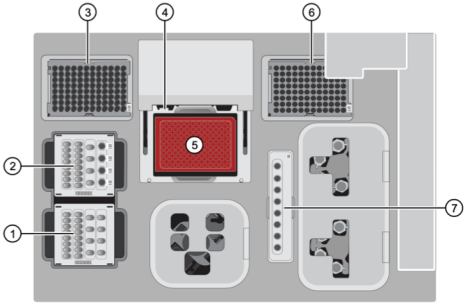
Position	Consumable
Solutions Station	Ion AmpliSeq™ Chef Solutions DL8 cartridge
Reagents Station	Ion AmpliSeq™ Chef Reagents DL8 cartridge
New Tip Station	New Ion AmpliSeq™ Tip Cartridge L8
Used Tip Station	Empty Tip Cartridge L8
Thermal Cycler Block	IonCode™ PCR Plate
Enrichment Station	Enrichment Cartridge

IMPORTANT

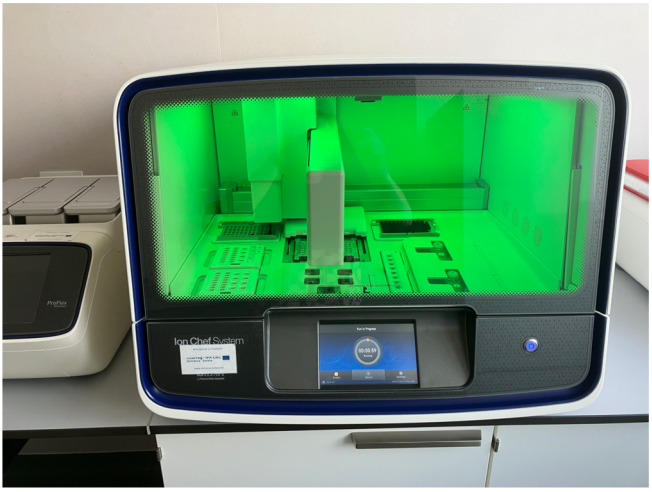
- Ensure proper cartridge orientation.
- Confirm uncapped reagent tubes in Positions A–D.
- Verify correct loading of primer pools in Positions A and B.
- Ensure PCR frame seal is correctly positioned.



① Position A (150 µL Primer Pool 1 at 2X concentration)
② Position B (150 µL Primer Pool 1 or 2 at 2X concentration)
③ Position C (Empty tube)
④ Position D (Output tube)



① Ion AmpliSeq™ Chef Solutions DL8 cartridge
② Ion AmpliSeq™ Chef Reagents DL8 cartridge
③ Ion AmpliSeq™ Tip Cartridge L8
④ PCR Frame Seal
⑤ IonCode™ 96 Well PCR Plate and PCR Plate Frame
⑥ Empty Tip Cartridge L8
⑦ Enrichment Cartridge



7. Starting the library preparation run

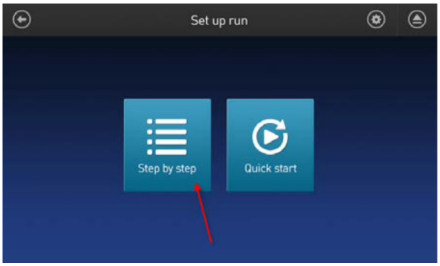
- From the Ion Chef™ touchscreen:
  - Select “Set up run”
  - Select “AmpliSeq”
- Perform Deck Scan verification.
- Confirm: server connection, Sample Set, barcode assignments.
- Select the appropriate amplification parameters.

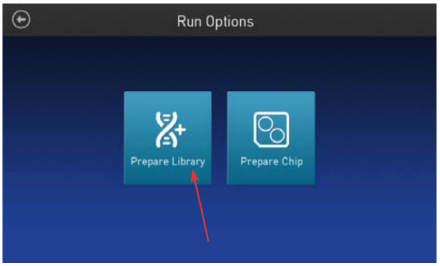
For HotSpot Panel v2:

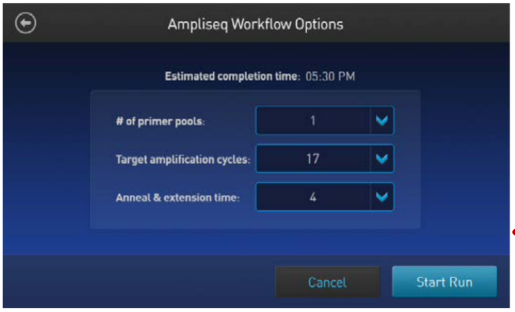
- Recommended amplification cycles depend on DNA quality and panel complexity.
- FFPE or low-quality DNA may require additional amplification cycles.

Start the run

Approximate runtime: 7-11 hours







Primer pairs per pool (excluding gene fusion primer pairs)[1]	Recommended number of amplification cycles (10 ng DNA/RNA, 3,000 copies)[2]		Anneal/Extension time[3]
	High quality DNA/RNA	Low quality DNA/RNA (FFPE DNA/RNA or cfDNA/RNA)	
Panels for gene fusion detection only	28	31	4 minutes
12–24	22	25	4 minutes
25–48	21	24	4 minutes
49–96	20	23	4 minutes
97–192	19	22	4 minutes
193–384	18	21	4 minutes
385–768	17	20	4 minutes
769–1,536	16	19	8 minutes
1,537–3,072	15	18	8 minutes

8. Automated library preparation steps

The Ion Chef™ System automatically performs:

8.1 Target Amplification

Multiplex PCR amplification of hotspot regions from cancer-associated genes.

8.2 Partial Primer Digestion

Enzymatic digestion removes residual primers from amplification reactions.

8.3 Adapter and Barcode Ligation

IonCode™ barcodes and sequencing adapters are ligated to amplified DNA fragments.

8.4 Library Amplification

Limited-cycle amplification enriches adapter-ligated fragments.

8.5 Purification and Normalization

Magnetic bead cleanup removes contaminants and normalizes library concentration. At completion, pooled barcoded libraries are recovered from Position D of the Reagents Cartridge. Libraries are approximately 100 pM total concentration.

9. Library recovery and storage

- Remove pooled libraries from Position D immediately after run completion.
- Cap tubes immediately to minimize evaporation.
- Store libraries:
  - 4–8°C for short-term storage,
  - −20°C for long-term storage.

Do not leave libraries inside the instrument beyond recommended holding time.

10. Template preparation (Templating)

Prepared libraries are used for automated template preparation on the Ion Chef™ System.

The templating workflow includes:

- library denaturation,
- emulsion PCR,
- enrichment of template-positive Ion Sphere™ particles (ISPs),
- chip loading preparation.

General templating workflow:

- Quantify pooled libraries if required.
- Prepare libraries according to template kit instructions.
- Load:
  - template reagents,
  - chips,
  - enrichment consumables,
  - libraries into Ion Chef™.
- Select appropriate templating workflow on touchscreen.
- Start automated template preparation.

The Ion Chef™ system performs:

- clonal amplification,
- ISP enrichment,
- chip loading preparation automatically.

11. Quality control

Recommended QC Steps

- Qubit™ library quantification
- Fragment analysis (Bioanalyzer/TapeStation)
- Assessment of library size distribution
- ISP enrichment evaluation after templating

Acceptance Criteria

- Minimal primer-dimer presence
- Expected amplicon size distribution
- Adequate library concentration
- Proper barcode assignment

12. Troubleshooting

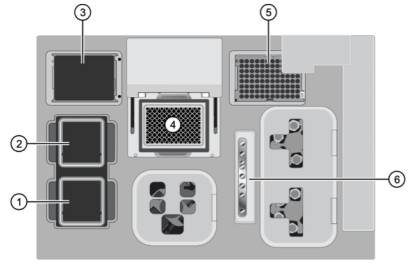
Observation	Possible Cause	Corrective Action
Low library yield	Low DNA quality	Repeat extraction or increase input
Uneven sequencing coverage	Poor amplification	Optimize DNA input
Excess primer dimers	Incomplete cleanup	Repeat purification
Failed Chef run	Incorrect cartridge loading	Verify deck setup
Low ISP enrichment	Incorrect library concentration	Requantify pooled library

13. Instrument cleaning

After every run:

- Remove all consumables.
- Initiate “Clean Ion Chef” procedure.
- Allow automated UV decontamination cycle to complete.

Routine cleaning minimizes cross-contamination risk and ensures reproducible sequencing performance.



① Solutions station
② Reagents station
③ New Pipette Tip station: move the empty Tip Cartridge to the Used Pipette Tip station
④ Thermal cycler sample block
⑤ Used Pipette Tip station
⑥ Enrichment station

14. Final output

The final output consists of:

- barcoded targeted DNA libraries,
- enriched template-positive ISPs,
- sequencing-ready chips for downstream analysis on Ion Torrent™ platforms.

The workflow is suitable for:

- somatic mutation profiling,
- translational oncology studies,
- precision medicine applications,
- targeted analysis of hotspot mutations in solid tumors.

Bench-Side Working Protocol for NGS - Automated Template Preparation, Chip Loading and Sequencing

Ion Chef™ System and Ion GeneStudio™ S5 Sequencing System Using Ion 530™ Chips

1. Purpose

This protocol describes the automated workflow for:

- template preparation,
- enrichment of template-positive Ion Sphere™ Particles (ISPs),
- automated chip loading,
- sequencing preparation,
- using the Ion Chef™ System and Ion GeneStudio™ S5 Sequencing System with Ion 530™ Chips.

The workflow is optimized for multiplexed targeted sequencing runs containing approximately 8–16 libraries/chip generated using Ion AmpliSeq™ workflows.
This protocol is intended for research use.

2. Materials and equipment

Reagents and consumables	Equipments
Ion 530™ Chips	Ion Chef™ Instrument
Ion 510™/520™/530™ Chef Reagents Cartridge	Ion GeneStudio™ S5 Sequencer
Ion S5™ Chef Solutions Cartridge	Vortex
Enrichment Cartridge v2	Minicentrifuge
Recovery Tubes v2	Calibrated pipettes
Recovery Station Disposable Lid v2	
Ion Chef™ Tip Cartridge	
Library Sample Tubes	
NaOH tube supplied with kit	
Chip Adapter and centrifuge buckets	

Input Material

- Prepared and pooled Ion AmpliSeq™ libraries
- Recommended multiplexing: 8–16 libraries/chip
- Libraries diluted according to sequencing workflow requirements

3. Library preparation before templating

- Quantify pooled libraries using fluorometric quantification.
- Confirm adequate library concentration and quality.
- Dilute libraries according to kit recommendations.
- Transfer 25 µL diluted pooled library into each Library Sample Tube.

IMPORTANT

- Libraries pooled in the same run must have comparable read lengths.
- Do not mix 200 bp and 400 bp libraries in the same Ion Chef™ run.

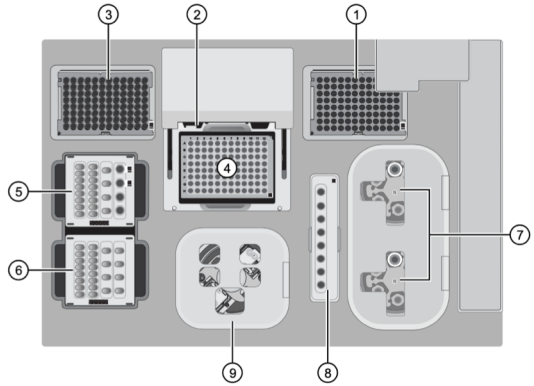


Figure 1 A schematic of a loaded Ion Chef™ Instrument

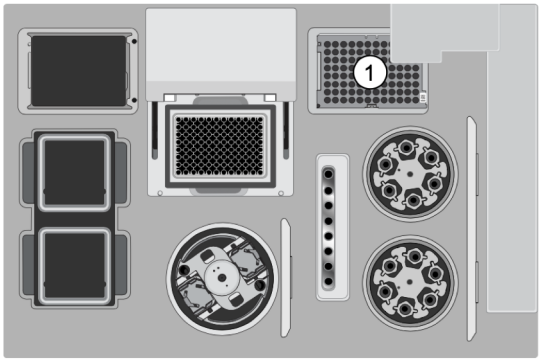
① Empty tip rack (move from new Tip Cartridge position)
② Frame Seal v2
③ New Tip Cartridge
④ PCR Plate and PCR Plate Frame
⑤ Ion 510™ & Ion 520™ & Ion 530™ Chef Reagents cartridge
⑥ Ion S5™ Chef Solutions cartridge
⑦ Recovery Tubes and Recovery Station Disposable Lid v2
⑧ Enrichment Cartridge v2
⑨ Chip Adapter/Chip assemblies

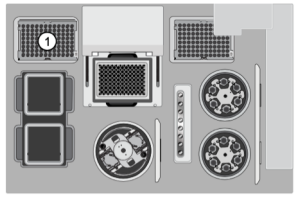
4. Loading the PCR plate and frame

- Insert the PCR Plate into the thermal cycler block.
- Position the PCR Plate Frame securely onto the thermal cycler sample block.
- Insert the Frame Seal v2 underneath the heated cover.

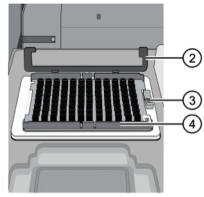
IMPORTANT

- Ensure the PCR Plate Frame is completely pressed down.
- Confirm that the Frame Seal v2 tabs contact the heated cover.

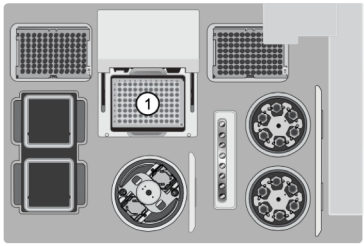




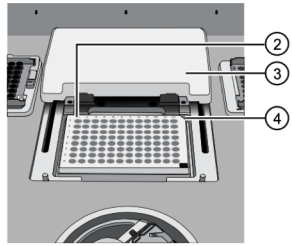
① New Pipette Tip Position
② Bracket



③ Catch
④ New Tip Cartridge v2



① Thermal cycler sample block
② Well A1



③ Cover
④ Keyed corner

5. Loading reagents and solutions

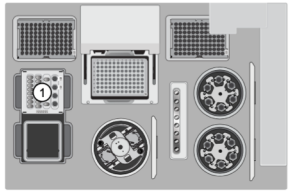
5.1 Reagents Cartridge

Thaw the Reagents Cartridge at room temperature for approximately 45 minutes.
Gently tap cartridge tubes to collect reagents at tube bottoms.
Load the Reagents Cartridge into the Reagents Station.
Load:

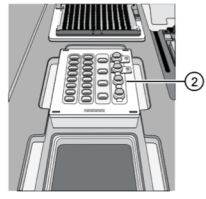
- Library tube in Position A
- Library tube in Position B
- NaOH tube in Position C
- Empty tube in Position D

IMPORTANT

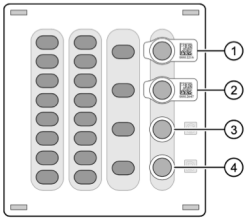
- Remove caps from all tubes before starting.
- Ensure barcode orientation is correct.



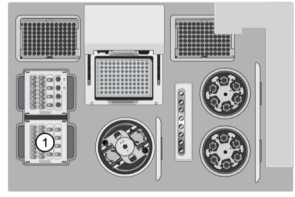
① Reagents station (4°C)



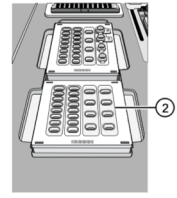
② Ion 510™ & Ion 520™ & Ion 530™ Chef Reagents cartridge



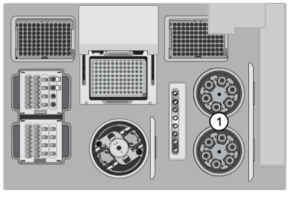
① Position A (Library)
② Position B (Library)
③ Position C (NaOH)
④ Position D (Empty tube)



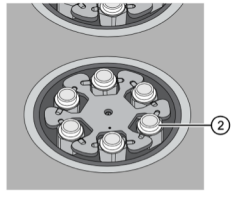
① Solutions station (room temperature)



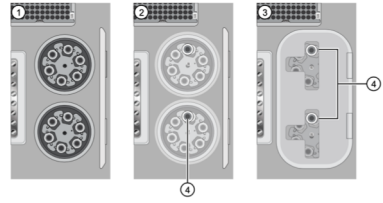
② Ion S5™ Chef Solutions cartridge



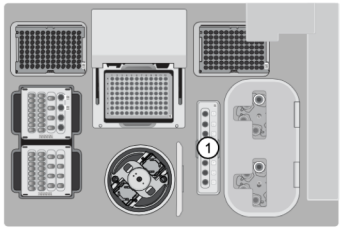
① Recovery centrifuges

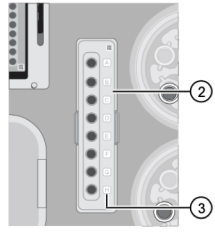


② Recovery Tube v2



① Recovery Tubes installed
② Recovery Station Disposable Lid v2 installed
③ Recovery centrifuge cover closed
④ Port





① Enrichment station
② Enrichment Cartridge v2
③ Lettering

5.2 Solutions Cartridge

- Gently mix the Solutions Cartridge.
- Load cartridge into the Solutions Station until securely locked.
- Ensure cartridge is level and properly seated on the deck.

6. Loading recovery tubes and enrichment cartridge

Recovery Tubes

- Insert Recovery Tubes into centrifuge buckets.
- Confirm load balance.
- Attach Recovery Station Disposable Lid v2.
- Close centrifuge covers securely.

IMPORTANT

- Ensure centrifuge buckets pivot freely outward.
- Do not obstruct centrifuge lids.

Enrichment Cartridge

- Load Enrichment Cartridge v2 into Enrichment Station.
- Press firmly until level with instrument deck.

IMPORTANT

- Cartridge lettering must be right-side-up.

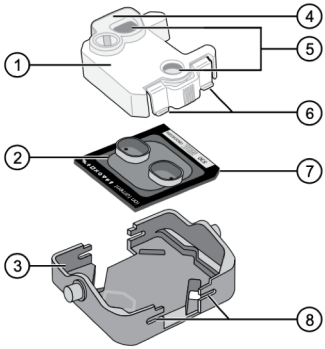
7. Chip loading preparation

7.1 Preparing the 530™ Chip

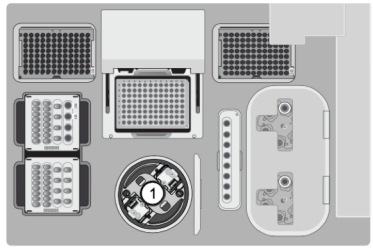
- Place Ion 530™ Chip into centrifuge bucket.
- Align keyed corners of chip and bucket.
- Attach Chip Adapter securely.
- Confirm adapter tabs are fully locked into bucket slots.

IMPORTANT

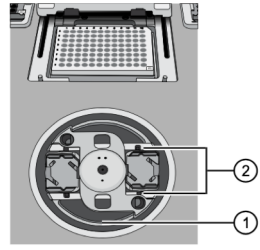
- Chip barcode must remain visible.
- Improper adapter attachment may cause loading failure.



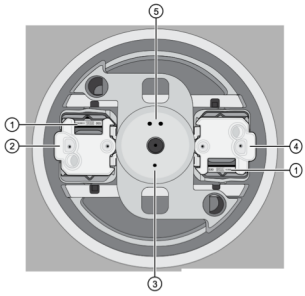
① Chip Adapter
② Ion Chip™
③ Bucket
④ Reservoir end of Chip Adapter
⑤ Ports (align with chip)
⑥ Flexible tabs
⑦ Keyed corner (align with bucket)
⑧ Slots



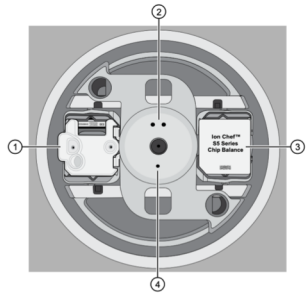
① Chip-loading centrifuge



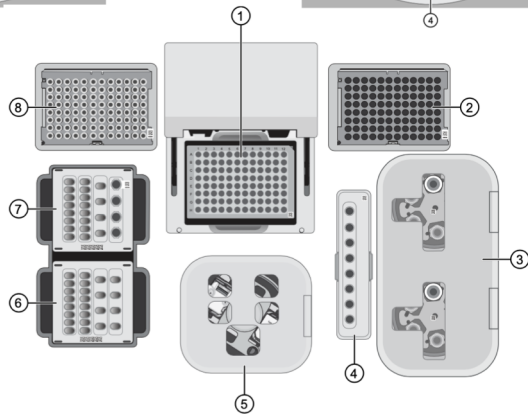
② Mounting grooves



① Chip barcode
② Chip position 1
③ Position 1 marker hole
④ Chip position 2
⑤ Position 2 marker hole



① Position 1 (chip)
② Position 2 marker holes
③ Position 2 (Chip Balance)
④ Position 1 marker holes



7.2 Loading Chip Centrifuge

- Insert adapter/chip assemblies into Chip-loading centrifuge.
- Confirm buckets are securely seated.
- Verify centrifuge load balance.

IMPORTANT

- Position 1 corresponds to Library Tube A.
- Position 2 corresponds to Library Tube B.

For single-chip runs: place chip in Position 1; use Ion Chef™ S5 Series Chip Balance in Position 2.

8. Pre-run checklist

Before starting the run, verify:

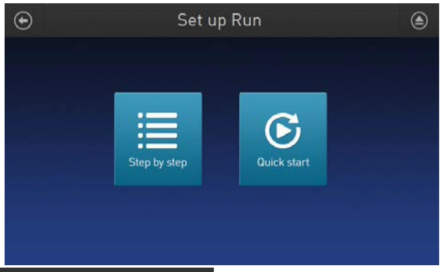
- PCR Plate and Frame installed correctly
- Frame Seal v2 positioned properly
- Reagents and Solutions cartridges loaded
- Recovery Tubes balanced
- Enrichment Cartridge inserted
- Chip adapters secured
- Tip cartridge installed
- All tubes uncapped
- Chips correctly positioned

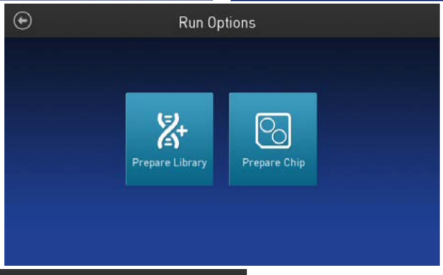
Perform manual verification even if Deck Scan is successful.

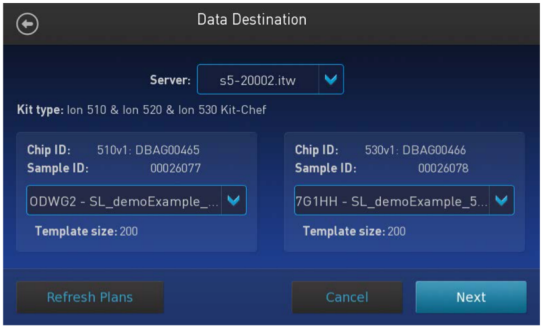
9. Starting ION CHEF™ run

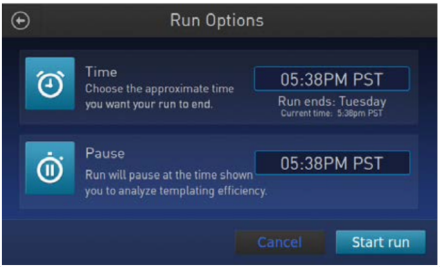
- On touchscreen:
  - Select “Set up run”
  - Select “Prepare Chip”
- Close instrument door correctly.
- Start Deck Scan.
- Verify: chip type, planned run, barcode assignments, kit compatibility.
- Select: immediate completion, or pause before chip loading for QC assessment.
- Run duration: approximately 13–15 hours depending on library size.


Ion Chef
Set up run
Notifications
Quick Start
Settings


Set up Run
Step by step
Quick start


Run Options
Prepare Library
Prepare Chip


Data Destination
Server: s5-20002.itw
Kit type: Ion 510 & Ion 520 & Ion 530 Kit-Chef
Chip ID: 510v1: DBAG00465
Sample ID: 00026077
ODWG2 - SL_demoExample_...
Template size: 200
Chip ID: 530v1: DBAG00466
Sample ID: 00026078
7G1HH - SL_demoExample_5...
Template size: 200
Refresh Plans
Cancel
Next


Run Options
Time
Choose the approximate time you want your run to end.
05:38PM PST
Run ends: Tuesday
Current time: 5:38pm PST
Pause
Run will pause at the time shown you to analyze templating efficiency.
05:38PM PST
Cancel
Start run


ionTorrent
Ion S5™ Sequencing Reagents


Ion GeneStudio S5
ionTorrent

10. Template preparation and chip loading

The Ion Chef™ System automatically performs:

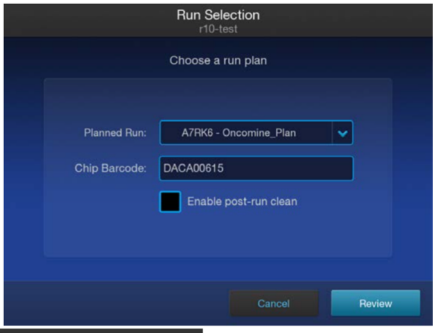
- library denaturation,
- emulsion PCR,
- clonal amplification,
- enrichment of template-positive ISPs,
- automated loading of ISPs onto Ion 530™ Chips.

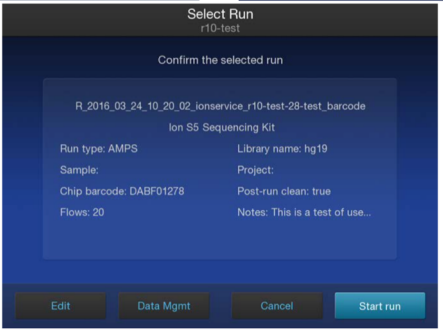
QC samples may be collected:

- before chip loading,
- or after run completion.

11. Sequencer initialization

- Initialize the Ion GeneStudio™ S5 Sequencer approximately 50 minutes before completion of chip loading.
- Verify sequencing reagents, waste container, calibration status, connectivity with Torrent Suite™.
- Sequencer initialization may be performed up to 24 hours before sequencing.





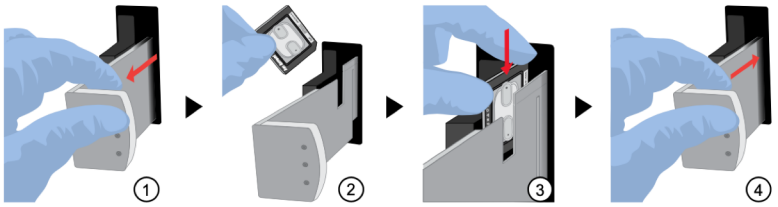
12. Chip transfer and sequencing

- Remove loaded Ion 530™ Chip from Ion Chef™ instrument.
- Inspect chip visually for bubbles or loading defects.
- Insert chip into Ion GeneStudio™ S5 Sequencer.
- Select corresponding sequencing run plan.
- Start sequencing run.

Monitor:

- ISP loading percentage,
- chip loading metrics,
- sequencing quality parameters.

① Touchscreen
② Power button
③ Ion S5™ Sequencing Reagents cartridge
④ Chip clamp
⑤ Ion S5™ Wash Solution bottle. The waste reservoir is located behind the Ion S5™ Wash Solution bottle (shown on the right).
⑥ Ion S5™ Cleaning Solution bottle
⑦ Waste reservoir



13. Quality control

Recommended QC parameters: ISP enrichment efficiency; Read number; Read length; Uniformity of coverage; Percentage of mapped reads; Barcode distribution across libraries.

Expected sequencing performance depends on: library quality, chip loading efficiency, panel complexity, number of multiplexed libraries.

14. Post-run procedures

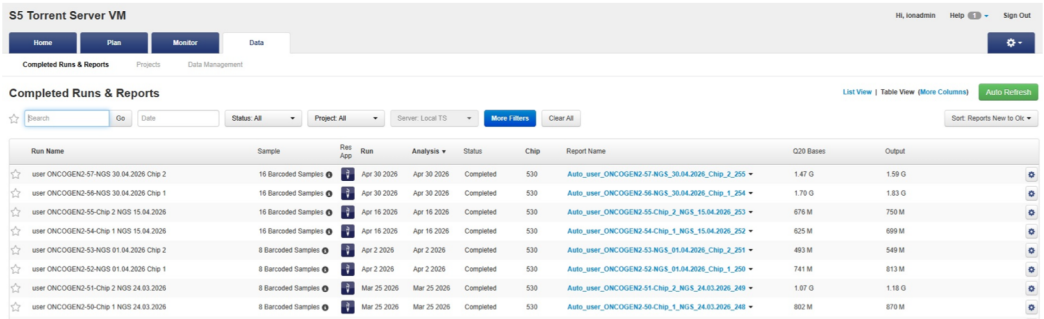
- Remove consumables from Ion Chef™ instrument.
- Perform instrument cleaning cycle.
- Dispose consumables according to laboratory biosafety procedures.
- Archive sequencing data and run reports.

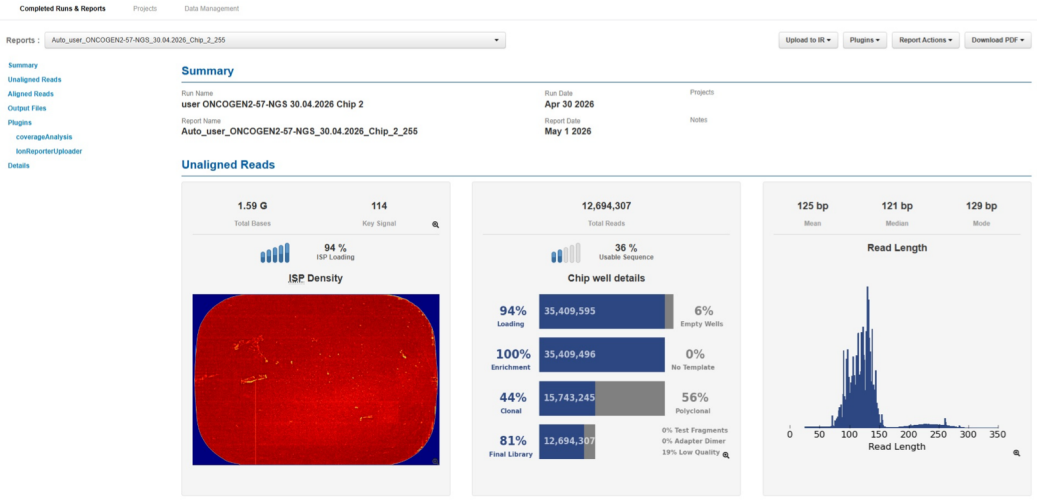
Routine maintenance minimizes cross-contamination and preserves sequencing consistency

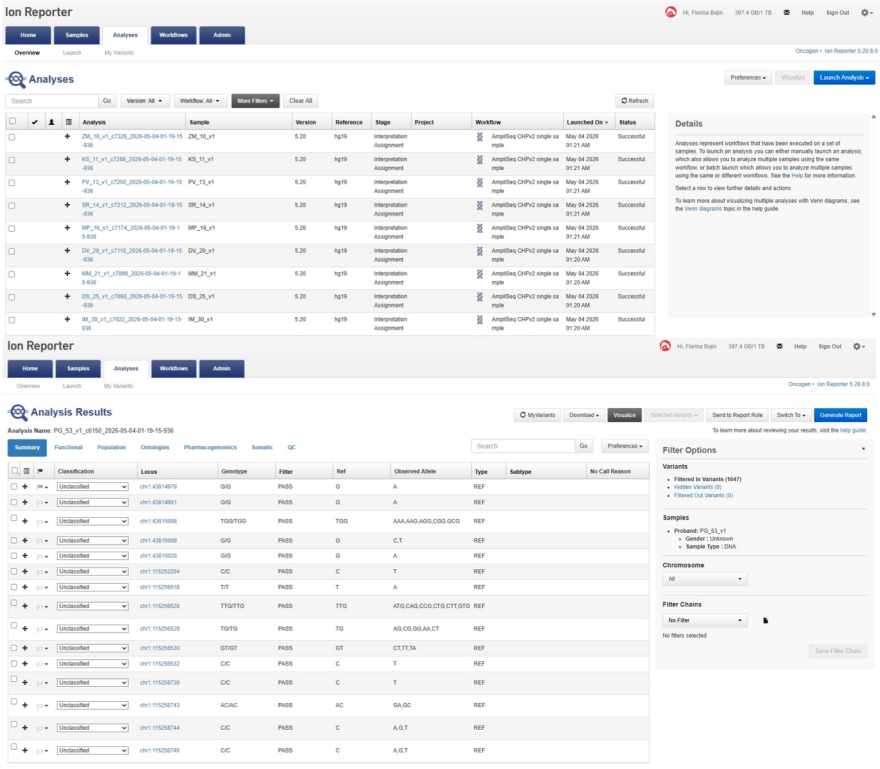
15. Final output

The workflow generates:

- enriched template-positive ISPs,
- sequencing-ready Ion 530™ Chips,
- FASTQ/BAM/VCF sequencing data suitable for:
  - targeted oncology sequencing,
  - HotSpot mutation analysis,
  - translational cancer research,
  - precision medicine applications.




Completed Runs & Reports
Projects
Data Management
Reports : Auto_user_ONCOGEN2-57-NGS_30.04.2026_Chip_2_255
Upload to IR
Plugins
Report Actions
Download PDF
Summary
Unaligned Reads
Aligned Reads
Output Files
Plugins
coverageAnalysis
IonReporterUploader
Details
Summary
Run Name
user ONCOGEN2-57-NGS 30.04.2026 Chip 2
Run Date
Apr 30 2026
Projects
Report Name
Auto_user_ONCOGEN2-57-NGS_30.04.2026_Chip_2_255
Report Date
May 1 2026
Notes
Unaligned Reads
1.59 G
Total Bases
114
Key Signal
94 %
ISP Loading
ISP Density
12,694,307
Total Reads
36 %
Usable Sequence
Chip well details
94%
Loading
35,409,595
6%
Empty Wells
100%
Enrichment
35,409,496
0%
No Template
44%
Clonal
15,743,245
56%
Polyclonal
81%
Final Library
12,694,307
0% Test Fragments
0% Adapter Dimer
19% Low Quality
125 bp
Mean
121 bp
Median
129 bp
Mode
Read Length
0 50 100 150 200 250 300 350
Read Length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Analyses
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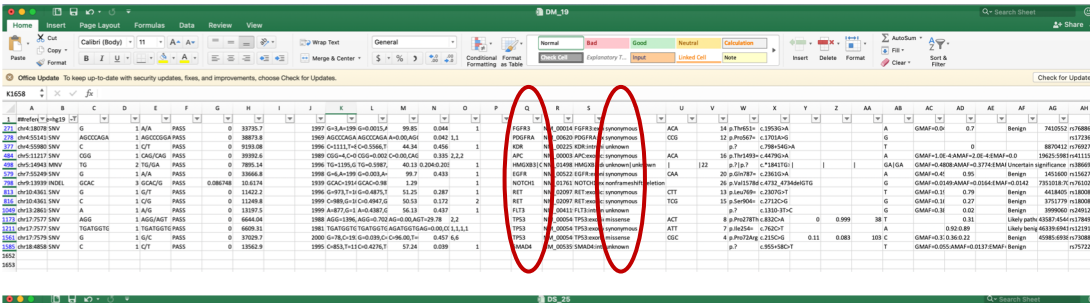
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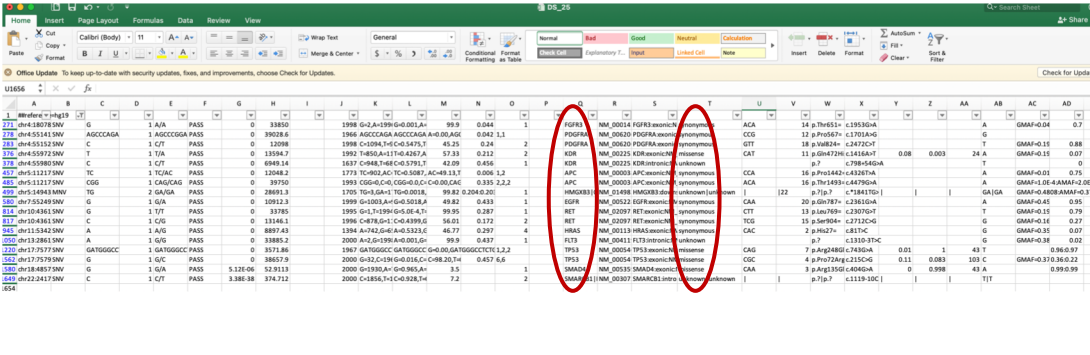
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Check for Updates
U1656

OncoGen Excellence Research Center

OncoGen — Centre for Gene and Cellular Therapies in the Treatment of Cancer
Str. Lidia nr. 48, 300210 Timișoara, România
Tel: +40 256 490 685 | oncogen@umft.ro | www.oncogen.ro

Patient: DS_25 **Cancer Type:** Colorectal Cancer **Report Date:** May 12, 2026

Immune Markers

B cells (Lymphocytes): Low | CD3 (Lymphocytes): Low | CD4 (CD3): Normal | CD8 (CD3): Normal | NK cells (Lymphocytes): Normal

Relevant Biomarker Summary

Variants classified as **Tier I** (Strong Clinical Significance) or **Tier II** (Potential Clinical Significance) per AMP/ASCO/CAP 2017.

Therapy Name = NCCN/ESMO guideline evidence Therapy Name = no guideline evidence yet

GENE / ALTERATION	TIER	THIS CANCER TYPE	OTHER CANCER TYPES
TP53 – R248Q	Tier II	5-Fluorouracil 5-Fluorouracil + Genistein Bevacizumab Bevacizumab + Oxaliplatin + Uft Celecoxib Cetuximab Cetuximab + Oxaliplatin + Uft Irinotecan Mdm2-P53 Interaction Inhibitors Nutlin-3 Olaparib Olaparib + Ku55933 Oxaliplatin P53 Inhibition P53 Reactivating Compounds P53 Restoration / P53 Pathway Restoration P53-Targeted Immunotherapy / Neoantigen T-Cell Therapy Radiotherapy Regorafenib Regorafenib + Other Targeted Therapy Combinations Rita	—

Variant Details (2)

2 variants (Tier I–III) ranked by AMP/ASCO/CAP 2017.

GENE / ALTERATION	CODING	LOCUS	VAF	FUNCTION	TIER
TP53 – R248Q	c.743G>A	chr17:7577528	—	missense	Tier II
SMAD4 – R135Q	c.404G>A	chr18:48575210	—	missense	Tier III

11 Tier IV variants (benign/synonymous) not shown.

Treatment Plan

Patient Summary

Molecular findings are most relevant for colorectal cancer biology, with a pathogenic TP53 R248Q hotspot mutation, likely loss-of-function SMAD4 R135Q, and an unspecified KDR variant at relatively high VAF suggesting possible VEGFR-pathway relevance. The remaining variants (FLT3, CSF1R, HMGXB3, SMARCB1, DERL3) are unspecified and/or very low VAF without actionable evidence; low B-cell and CD3 lymphocyte counts may increase caution with myelosuppressive or heavily toxic regimens but do not create a specific targeted-therapy indication from the supplied evidence.

Associated Therapies

#1 Bevacizumab

Supporting Genes	KDR, TP53
Evidence Strength	Moderate Evidence
Key PMIDs	22273502, 22139032, 22539090, 28315202, 36601631, 21791631, 23579861, 31366114, 23464465, 15998951
Monitoring	Monitor blood pressure, urine protein, renal function, bleeding/thrombotic events, wound-healing complications, GI perforation risk, and CBC/chemistry when combined with chemotherapy.
Rationale	Bevacizumab has convergent supportive evidence from both KDR and TP53 in colorectal cancer. The KDR alteration makes VEGF/VEGFR-pathway inhibition biologically plausible, although the exact variant is unspecified, and TP53 DNA-binding domain missense mutations such as R248Q have been associated in some cohorts with favorable outcomes on bevacizumab-based therapy. This is stronger and more internally consistent than anti-EGFR or fluoropyrimidine-focused approaches, which are weakened here by SMAD4- and TP53-associated resistance signals.

#2 Bevacizumab + Chemotherapy

Supporting Genes	KDR, TP53, SMAD4
Evidence Strength	Moderate Evidence
Key PMIDs	31579770, 34359764, 20072938, 31366114, 24384683, 12237773, 12584741, 17982676, 32457882, 29738772, 19189638, 28851987, 36039738, 21616090, 15856030
Monitoring	In addition to bevacizumab monitoring, follow CBC, CMP, mucositis/diarrhea, neuropathy if oxaliplatin-containing, hand-foot syndrome depending on fluoropyrimidine used, and infection risk given low B cells/CD3 lymphocytes.
Rationale	This regimen-level option is supported by colorectal cancer literature and is biologically aligned with the KDR/VEGFR2 pathway signal. However, confidence is moderated because both SMAD4 and TP53 are associated with reduced benefit from 5-fluorouracil, and TP53 also carries a resistance signal for oxaliplatin; therefore, if this regimen is used, the anti-VEGF component is the strongest rationale while the chemotherapy backbone may be less favorable biomarker-wise.

#3 Ramucirumab + Chemotherapy

Supporting Genes	KDR, SMAD4, TP53
Evidence Strength	Moderate Evidence
Key PMIDs	35117928, 29228087, 34359764, 24384683, 12237773, 12584741, 17982676, 32457882, 29738772, 19189638, 28851987, 36039738, 21616090, 15856030
Monitoring	Monitor hypertension, proteinuria, bleeding, thrombosis, GI perforation, liver function, CBC, diarrhea, and regimen-specific chemotherapy toxicities. Assess performance status carefully before combination treatment.
Rationale	Ramucirumab directly targets VEGFR2/KDR and has supportive colorectal cancer evidence as a clinically relevant antiangiogenic regimen, particularly in later-line metastatic settings. The KDR alteration supports pathway relevance, but the exact variant is unknown, and the chemotherapy backbone remains potentially attenuated by SMAD4/TP53-associated fluoropyrimidine resistance signals.

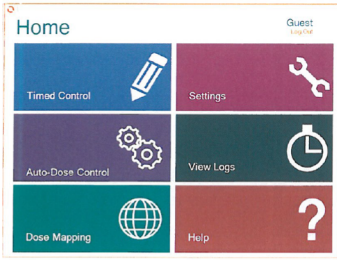
#4 Ramucirumab

Supporting Genes	KDR
Evidence Strength	Moderate Evidence
Key PMIDs	35117928, 29228087, 19513949, 27733377, 33769095

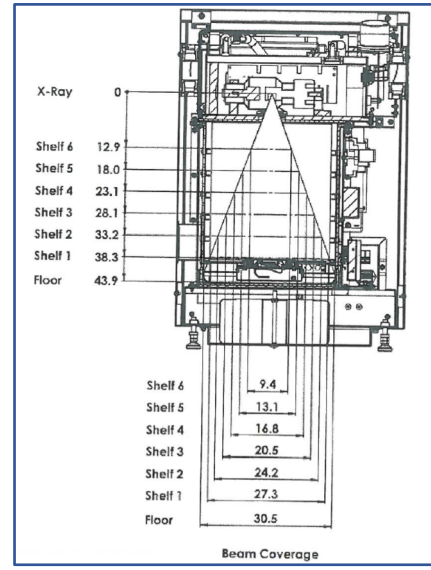
Working Protocol for Cells and Tissues Sterilization - CellRad+

Technical description of CellRad+

Energy Range: 20 - 150kV
Tube Current: 0.1 - 6.25mA
Tube Power: 940W
Focal Spot Size: 2.5mm x 2.5mm
Inherent Filtration: 3.37mm Dielectric Oil, 2.38mm Ultem, 1.5mm Glass, 0.8mm Beryllium Beam Angle: 40° Divergence
Maximum Coverage: 12" (30cm)
Source to Shelf Distance (SSD): 17" (44cm)
Exposure Time: 5 seconds to 180 minutes (in 1 second increments) Continuous Operation
Power Requirements: 220-240VAC, 50/60Hz, 1500W Integrated Closed Loop Heat Exchanger for Cooling Tube Turntable: Electrically Operated, 2 RPM
Environmental conditions: This equipment has been designed for indoor use only.
Storage/operating temperature: 5 to 40° C.
Optimum room temperature when operating at maximum power: 23° C.
Relative humidity: < 80%. Pollution degree: 2
Overvoltage category: II



Shelf positions and beam coverage



Basic Operating Procedure

1. Turn the key switch to the ON position. The computer and software will automatically power on

NOTE: The key will be stuck in the ON position

2. Press the Login button to log in as a guest or select a user name and enter the assigned password (if required).
3. Before performing the first procedure of the day, a warm-up must be performed. After the warm-up is complete (approx. 30 minutes), the user can proceed to the main screen.
4. Dose Quality Check. Dose QA is a method of verifying that the X-ray and dosimeter are operating within the proper parameters. Dose QA is not required and the system will operate normally. However, without performing a QA, the dose measurements cannot be verified. If Dose QA fails, check that there is nothing in the chamber, that the shelf is positioned correctly, and that the aluminum filter is in place.
5. Place a sample in a container and place it inside the CellRad+ system. The irradiation area is designated by the circular outlines on the turntable.
6. From the menu, select Timed Control. The user can enter the time, kilovolts (kV), and milliamperes (mA). The device will calculate and display the delivered dose directly.
7. To begin the exposure, select a program preset or manually set the required values.
8. Press the green X-ray button to begin the procedure. The system will begin a 5-second pre-warning, followed by a countdown. The red X-Ray On light will turn on when the exposure begins.
9. Wait until the timer countdown is complete. 10. Remove the specimen from the turntable.

Repeat steps 5 through 10 for additional samples.

To turn the system off, turn the key to the OFF position. The system will automatically shut down the software and turn off the system. The CellRad+ system will enter a cool down mode. The cool down time will vary depending on usage.
Never connect or disconnect the communication and/or power cables while the system is powered. The software must be shut down according to the procedure described in section 2.5.0. of the User's Manual;

Warm-up should be performed daily to ensure optimal system performance. The software monitors the time between uses and increases or decreases the warm-up time accordingly. Please refer to the warm-up procedure described in section 4.2.0. of the User's Manual.

When the system is powered on, check that the fans are operational and that air is flowing out of the unit. The fans are located on the back and sides of the system.

The ventilation openings must be clear of any obstructions or walls to allow the system to cool properly.

Configuring multiple users and passwords is done from the main menu, after an administrator account has been set up.

Daily Inspection

Each day, before use, the operator should perform the following visual inspection to verify the integrity of the system:

- Inspect the irradiation chamber and exterior of the instrument, visible components and cables for signs of damage;
- Verify that the door closes properly and that there are no loose screws or hardware on the locking mechanism;
- Verify that the power LED lights up when the system is turned on;
- Verify that the display lights up and that the software starts.

Internal Cleaning

Wipe the internal chamber periodically with a damp cloth and mild detergent. The internal chamber can be disinfected with isopropyl alcohol.

External Cleaning

Wipe the system periodically with a damp cloth and mild detergent. To prevent scratching, do not use abrasive or harsh cleaning products.

NOTE: Do not allow moisture to come into contact with the electrical components of the unit.

Annual Maintenance

To ensure that the CellRad+ system continues to operate at optimum performance, annual maintenance by a certified service engineer is recommended to include, but is not limited to:

- Standard warm-up and testing procedures
- Safety system checks
- Dosimetry checks
- Office radiation survey

Due to the penetrating properties of ionizing radiation and their ability to inactivate microorganisms, ionizing radiation is used for many different purposes including, virus inactivation for research laboratories, as well as to sterilize or reduce the microbial bioburden of many different types of products such as medical devices, packaging, cosmetics, foods, and agricultural products. It is also used to alter the properties of a wide variety of polymers through numerous chemical reactions.

Ionizing radiation such as X-rays and gamma rays can easily penetrate most tissues, and kill bacteria by causing irreparable DNA damage. Many Gram negative bacteria such as E. coli, Salmonella, and P. aeruginosa can be effectively killed by X-rays. Results also showed that X-rays of lower energies were effective in inactivating bacterial spores.

Advantages of X-ray sterilization

- Excellent penetration and improved Dose Uniformity Ratio (DUR).
- Fast, efficient processing and flexibility.
- Environmental safety, no specialized chemicals or disposal concerns.
- Compatible with plastics, glassware and some biological materials

The use of CellRad+ in research and medical activities

- In the sterilization of tissues
- In the sterilization of culture media
- The inhibition of the growth and development of certain fungi
- The sterilization of various plastic materials

Working SOP – Liberty Blue 2.0, Standard Fmoc-SPPS

Purpose: routine Fmoc solid-phase peptide synthesis (SPPS) protocol on a CEM Liberty Blue 2.0 microwave peptide synthesizer

IMPORTANT

- Use **10% piperidine in DMF** for 0.1 mmol syntheses (not 20%).
- Use **0.2 M amino acid solutions**, **1.0 M Oxyma**, and **1.0 M DIC** for 0.1–0.5 mmol syntheses.
- Liberty Blue is specifically optimized for **DIC/Oxyma chemistry**.
- Standard deprotection and coupling are performed at **90°C**, not 75°C.
- Histidine derivatives other than His(Boc) require **50°C coupling**.
- Arginine should be **double coupled** by default.

For most academic peptide synthesis projects (10–40 amino acids, 0.1 mmol scale), the most robust Liberty Blue 2.0 setup is:

- Rink Amide ProTide resin
- 0.2 M amino acids in DMF
- 1.0 M Oxyma
- 1.0 M DIC
- 10% Piperidine in DMF
- 90°C coupling
- 90°C deprotection
- Double coupling for Arg and difficult regions
- Fmoc-His(Boc)-OH whenever histidine is present

These conditions match the manufacturer's current Liberty Blue 2.0 recommendations and typically provide excellent crude purities for peptides up to ~30–40 residues.

1. Recommended chemistry

Resin

For C-terminal amides: Fmoc-Rink Amide ProTide resin
For C-terminal acids: preloaded Wang resin
Recommended mesh:

- 100–200 mesh
- 75 µm

Never use 200–400 mesh resin because it may clog the frit.

2. Reagent preparation

Main Solvent: DMF (CEM preferred solvent)

Deprotection Solution

For 0.1 mmol scale: 10% Piperidine in DMF
Alternative: Piperazine/NMP system if desired.

Amino Acids

Prepare each amino acid: 0.2 M Fmoc-AA-OH in DMF
Example: 1 mmol amino acid dissolved in 5 mL DMF.
Store: ≤2 weeks
Histidine and cysteine solutions should be checked frequently for precipitation.

Activator 1

Oxyma Pure, 1.0 M in DMF

Activator 2

DIC, 1.0 M in DMF

3. Instrument setup

Reaction Vessel

For 0.1 mmol synthesis: 30 mL reaction vessel
Recommended resin amount: typically 150–300 mg depending on loading.
The 30 mL vessel is approved for scales up to 0.2 mmol.

4. Loading reagents

Load:

- AA solutions into amino-acid positions
- Oxyma into ACT1
- DIC into ACT2
- DMF into main solvent bottle
- Piperidine solution into deprotection bottle

Prime lines using the Change Bottle procedure.

5. Resin loading

- Remove RV.
- Disconnect drain union.
- Add weighed dry resin.
- Reassemble vessel.
- Insert vessel into microwave cavity.
- Insert fiber optic probe completely to bottom of thermowell.

Failure to fully insert the probe may cause severe overheating and poor synthesis.

6. Method creation

For routine synthesis:

Scale - 0.1 mmol

Resin Type - Preloaded resin (if using preloaded resin) or Not Preloaded (if first amino acid must be loaded)

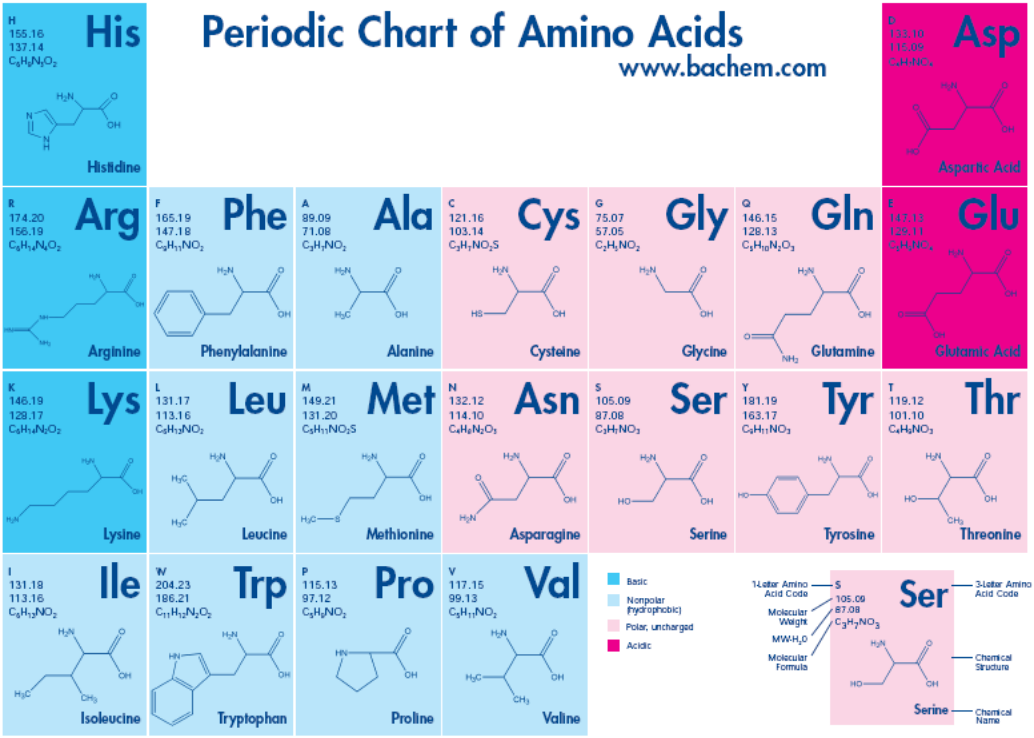
Final Deprotection - choose: remove terminal Fmoc or retain terminal Fmoc according to peptide design.

7. Standard coupling cycle

The Liberty default cycle is already optimized and should be used whenever possible.

Coupling Chemistry: 5 equivalents amino acid; 5 equivalents Oxyma; 5 equivalents DIC

Microwave Conditions: target - 90°C; heating profile - reach 90°C within 20–30 s, maintain ≥100 s for standard 0.1 mmol coupling cycles.



8. Standard deprotection cycle

Reagent: 10% Piperidine/DMF

Microwave Conditions: target - 90°C, heating profile - reach temperature in 20–30 s, maintain ≥45 s (according to CEM specifications).

9. Special amino acids

Arginine

Always use double coupling (recommended by CEM).

Histidine

Preferred derivative: Fmoc-His(Boc)-OH to minimize epimerization.

For other His derivatives: couple at 50°C, not 90°C.

Difficult Regions

Use Double Coupling for residues after position 25, Val/Ile/Leu clusters, hydrophobic stretches, deletion-prone regions (as recommended by CEM).

10. Long peptides (> 25 aa)

Recommended:

- PEG-PS (ProTide) resin
- low loading resin
- double coupling throughout difficult regions

11. Cleavage

Liberty Blue does not perform cleavage.

Recommended Cleavage Cocktail

TFA/TIS/H_2O/DODT

92.5 : 2.5 : 2.5 : 2.5

Conditions

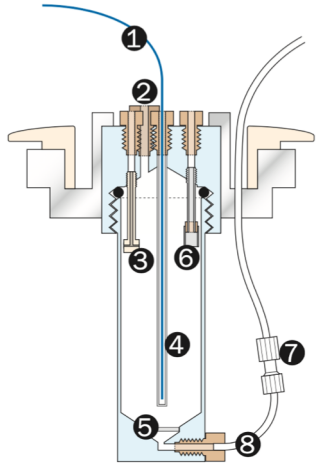
Option 1: 40–42°C, 30 min (using Razor)

Option 2: room temperature, 3 h

12. Post-cleavage workup

- Filter resin.
- Collect TFA solution.
- Precipitate in cold ether.
- Centrifuge.
- Wash pellet 2–3× with ether.
- Dry peptide.
- Analyze by LC-MS.
- Purify by RP-HPLC if required.

Reaction Vessel Components



	Item
1	Fiber Optic Probe
2	Vent Line
3	Wash Line*
4	Thermowell
5	Frit
6	Reagent Line*
7	Union
8	Drain Line

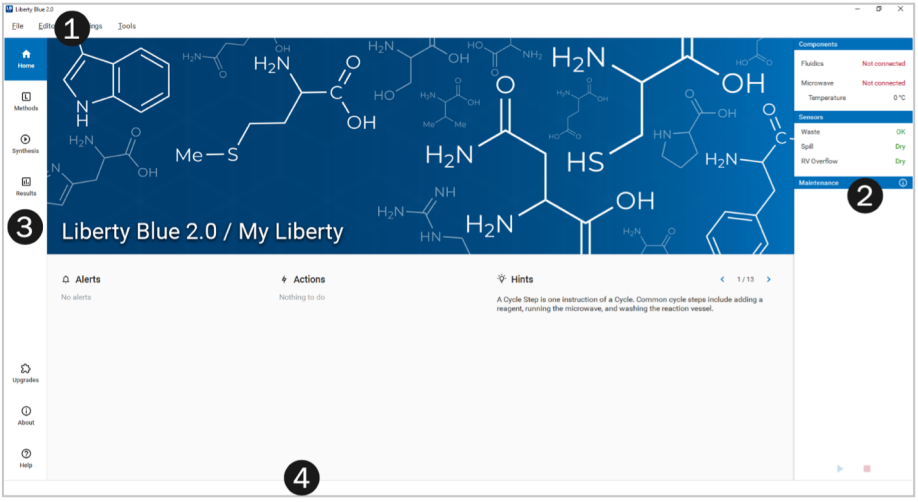
The Liberty Blue 2.0 is the latest generation of automated microwave peptide synthesizer. Built on CEM's flexible Discover®microwave platform, the Liberty Blue 2.0 is capable of synthesizing peptides faster and more efficiently than conventional synthesizers, thanks to the system's patented circular microwave cavity. The Liberty Blue 2.0's efficient Flex-AddTM technology eliminates the need for sample loops and allows infinite volume delivery options for a wide range of synthesis scales. The Liberty Lite 2.0 is compatible with the with the HT4 option and the Liberty Blue 2.0 is compatible with the HT4TM or HT12. The HT modules provide increased throughput, comparable to that of conventional high throughput systems, but with considerably better purity.

13. Liberty Software overview

The operation of the Liberty Blue 2.0 is controlled through the Liberty Software. The Liberty Blue 2.0 includes an external computer controller for running the Liberty Software. This computer is connected to the Liberty Blue 2.0 and the Discover microwave through an Ethernet connection or wireless router.
The software operates best with a display resolution of 1920x1080 or larger, and a display scaling of 100%.

Software Layout Overview

The software consists of 4 main sections: Drop-down Menus, System Status Panel, Navigation Tabs and Run System Status.



	Item	Description
1	Drop-down Menus	Provides access to instrument features and functionality.
2	System Status Panel	Shows communication status of components, current temperature and system pressure readings, microwave method parameters, sensor status for the waste container, spill tray, and reaction vessel overflow sensors. The Maintenance Log provides a list of routine maintenance operations.
3	Navigation Bar	Allows the user to navigate between the most common actions - creating and running a Liberty Method and then viewing the results. Instrument specific information and documentation can be viewed along with optional upgrades.
4	Run Status Bar	Shows the status of the current synthesis/method.

Gene Expression Analysis in Solid Tumor Samples Using the QuantStudio™ 5 Real-Time PCR System

Laboratory Platform: Real-Time PCR and Molecular Diagnostics Core Facility
Instrument: QuantStudio 5 Real-Time PCR System
Application: Relative gene expression analysis of solid tumor samples using RT-qPCR
Method: Reverse Transcription Quantitative PCR (RT-qPCR)

1. Purpose

This Standard Operating Procedure (SOP) describes the workflow for quantitative gene expression analysis in solid tumor samples using the QuantStudio™ 5 Real-Time PCR System. The procedure includes RNA extraction quality assessment, cDNA synthesis, quantitative PCR setup, instrument operation, quality control, and data analysis using the comparative Ct (ΔΔCt) method.
This protocol is intended for translational oncology research, biomarker validation studies, and precision medicine applications.

2. Principle of the method

Gene expression profiling by RT-qPCR is based on the quantification of messenger RNA (mRNA) transcripts present in tumor tissues.

The workflow consists of:
Tumor Tissue → RNA Isolation → cDNA Synthesis → qPCR Amplification → Data Analysis

During amplification, fluorescent signals generated by sequence-specific probes or intercalating dyes are measured in real time. The cycle threshold (Ct) value reflects the abundance of the target transcript.
Relative expression levels are calculated after normalization to endogenous reference genes and comparison with a calibrator sample.

3. Sample requirements

Accepted Sample Types

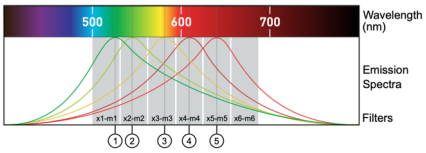
- Fresh frozen tumor tissue
- RNAlater®-preserved specimens
- FFPE-derived RNA (validated assays recommended)
- Tumor biopsies
- Surgical resection specimens

RNA Quality Requirements

Parameter	Acceptance Criteria
A260/A280	1.8–2.1
A260/A230	>1.8
RNA Concentration	≥20 ng/µL
RIN Score	≥7 preferred

RNA samples should be stored at −80°C for long-term storage, on ice during processing. Repeated freeze-thaw cycles should be avoided.

4. Workflow overview



Step 1
RNA Isolation and Quality Control
↓
Step 2
DNase Treatment
↓
Step 3
Reverse Transcription (cDNA Synthesis)
↓
Step 4
qPCR Reaction Preparation
↓
Step 5
QuantStudio™ 5 Run
↓
Step 6
Quality Assessment
↓
Step 7
ΔΔCt Analysis and Reporting

5. RNA extraction and quality control

RNA Isolation

Total RNA should be isolated using validated commercial kits according to the manufacturer's instructions.
Recommended controls:

- Extraction blank control
- Positive extraction control

Quality Assessment

RNA quantity and purity should be evaluated using:

- Spectrophotometry (NanoDrop™)
- Fluorometric quantification (Qubit™)
- Electrophoretic analysis (Bioanalyzer or TapeStation)

Quality Acceptance Criteria

RNA samples meeting all quality criteria proceed to reverse transcription. Samples with excessive degradation should be excluded or re-extracted.

6. cDNA synthesis

Input Material

500–1000 ng total RNA per reaction

Reverse Transcription Reaction

Prepare reverse transcription reactions according to the manufacturer's instructions. Following synthesis, cDNA should be diluted according to assay requirements and stored at: −20°C (short term); −80°C (long term)

Typical Thermal Program

Step	Temperature	Time
Primer Annealing	25°C	10 min
Reverse Transcription	42–50°C	30–60 min
Enzyme Inactivation	85°C	5 min
Hold	4°C	∞

7. qPCR reaction setup

Recommended Detection Chemistries

TaqMan™ Gene Expression Assays

Advantages:

- High specificity
- Multiplex capability
- Reduced non-specific amplification

SYBR™ Green Assays

Advantages:

- Lower cost
- Flexible assay design
- Requires melt curve analysis.

Standard 20 µL Reaction

Component	Volume
2× Master Mix	10 µL
Gene Expression Assay	1 µL
cDNA Template	2 µL
Nuclease-Free Water	7 µL
Total Volume	20 µL

Controls

Each experiment should include:

Negative Controls

- No Template Control (NTC)
- No Reverse Transcriptase Control (No-RT)

Positive Controls

- Known expressing sample
- Reference standard

Replicates

- Technical triplicates
- Biological replicates

8. Plate design

The QuantStudio™ 5 Real-Time PCR System utilizes a 96-well optical plate format. The plate should be loaded with well A1 positioned in the upper-left corner and oriented according to manufacturer recommendations.

Recommended Plate Layout

Sample Type	Wells
Tumor Samples	Triplicate
Calibrator Sample	Triplicate
Reference Gene Assays	Triplicate
NTC Controls	Triplicate
No-RT Controls	Triplicate

Recommended Reference Genes

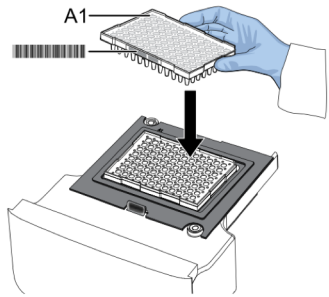
Reference genes must be validated for each tumor type.
Commonly used genes include: GAPDH, ACTB, HPRT1, RPLP0, TBP. At least two reference genes are recommended for clinical research studies.

9. QuantStudio™ 5 operation

Instrument Preparation

Before each run:

- Verify instrument status
- Confirm calibration validity
- Check optical components
- Review maintenance log



The QuantStudio™ 5 uses fluorescence-based detection within a fixed 96-well block configuration. The manufacturer recommends periodic verification of:

- ROI/Uniformity Calibration
- Background Calibration
- Dye Calibration

Calibration status should be reviewed regularly as part of quality assurance procedures.

Plate Loading

- Centrifuge plate briefly.
- Inspect wells for bubbles.
- Seal using optical adhesive film.
- Load plate into instrument.
- Confirm correct orientation.
- Close instrument drawer.
- Start experimental run.

10. Thermal cycling conditions

TaqMan™ Assays

Initial Activation
95°C – 10 min
Amplification (40 Cycles)
95°C – 15 sec
60°C – 60 sec

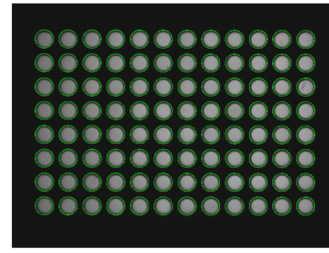
SYBR™ Green Assays

Initial Activation
95°C – 10 min
Amplification (40 Cycles)
95°C – 15 sec
60°C – 60 sec

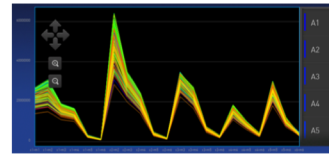
Melt Curve Analysis

Required for specificity verification.

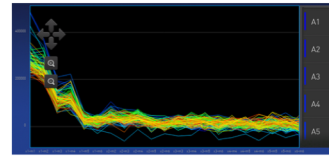
Green circles around all wells and bright well centers.



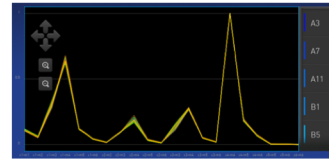
Signals from each well following a uniform trend.



Few, if any, signals with abnormally high fluorescence.



Signals from each well following a uniform trend, and each dye peaks at the correct filter.



11. Data analysis

Quality Control Criteria

Parameter	Acceptance
Replicate SD	<0.3 Ct
NTC Amplification	None
Melt Curve Peak	Single Peak
PCR Efficiency	90–110%

Relative Quantification

Step 1

ΔCt = Ct(Target) − Ct(Reference)

Step 2

ΔΔCt = ΔCt(Sample) − ΔCt(Calibrator)

Step 3

Fold Change = 2^(-ΔΔCt)

Interpretation:

Fold Change >1 = Upregulation

Fold Change <1 = Downregulation

12. Reporting of results

The final report should contain:

Sample Information: Sample ID, Tumor type, RNA concentration, RNA quality metrics

Experimental Parameters: Target genes, Reference genes, Assay chemistry, Instrument settings

Results: Ct values, ΔCt values, ΔΔCt values, Fold change values, Statistical analysis. Representative amplification plots and expression graphs should be included whenever possible

13. Quality assurance and data management

To ensure reproducibility:

- Use RNase-free consumables.
- Perform all analyses in triplicate.
- Include appropriate controls in every run.
- Document instrument maintenance and calibration records.
- Archive raw data files and analysis reports.

QuantStudio™ run files and associated experimental data should be exported and securely stored according to institutional data management policies.

14. Applications in oncology

The QuantStudio™ 5 platform can be applied to:

- Cancer biomarker validation
- Molecular tumor characterization
- Therapy response monitoring
- Immune-oncology studies
- Precision medicine programs
- Gene signature validation
- Translational cancer research

The method provides rapid, sensitive, and reproducible quantification of gene expression in clinical and research oncology settings.

This booklet presents a compilation of experimental and analytical protocols developed within the RO-SRB–funded project *Cross-border cooperation to foster the resilience of clinical management in cancer patients by establishing best practices in personalized molecular-based diagnostics, treatment, and long-term care* - RORS00040, during 18 months implementation period.

The current document represents Version 2.0 of the protocol collection and includes additional methodologies, optimized workflows, and updated analytical procedures developed during the first six months of the second year of project implementation. The protocols were refined based on ongoing experimental activities, inter-institutional collaboration, and the integration of newly generated research data within the project framework.

Version 2.0 / Final public research deliverable

The protocols included in this booklet are intended exclusively for research and scientific use within the framework of the RO-SRB project and associated collaborative research activities.

BETTER diagnosis, care, lives!