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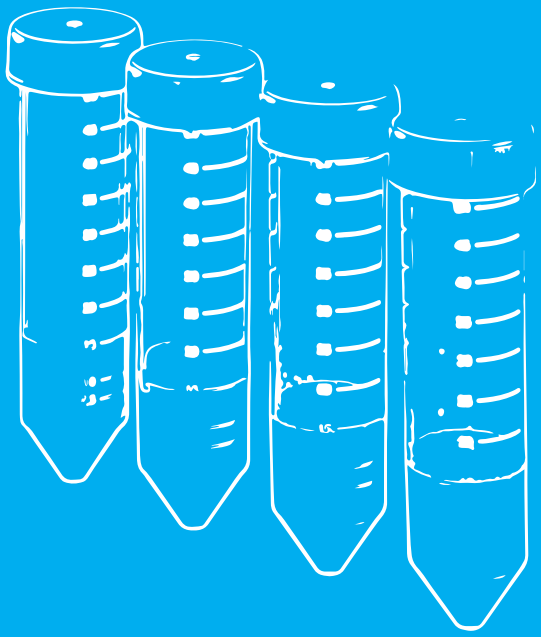
INSTRUCTIONS FOR USE



ONCOQUICK®

Instruction Manual

English Version

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greiner BIO-ONE

TABLE OF CONTENT

1/ INTENDED USE:

OncoQuick® is designed for the enrichment of disseminated circulating tumor cells from whole blood and has been optimized for 15 mL to 30 mL of anticoagulated whole blood.
Note: The kit is intended for researchuse only. It shall not be used in diagnostic procedures.

2/ INTRODUCTION:

In past years there has been an increasing clinical interest in detecting circulating tumor cells in bone marrow and whole blood and to integrate the monitoring of minimal residual disease (MRD) in tumor staging and prognosis. Tumor cells can be identified and enumerated quantitatively with a sensitivity that is limited only by the volume of blood used. **OncoQuick®** has been designed to enrich circulating tumor cells from up to 30 mL of whole blood. **OncoQuick®** employs a liquid separation medium that has been optimized for the specific enrichment of circulating tumor cells solely based on their buoyant density under appropriate conditions. The enrichment principle does not rely on the expression of specific membrane antigens such as EpCAM or ErbB-2 and yields bead- and antibody-free tumor cells. The entire enrichment procedure can be completed within 45 minutes yielding not more than approx. 104 total mononuclear cells and a total enrichment factor of up to 6 log-units.

3/ PRODUCT DESCRIPTION AND PRINCIPLE OF THE ENRICHMENT PROCEDURE:

The **OncoQuick®** kit is comprised of ten 50 mL polypropylene centrifugation tubes. For ease of use, each centrifugation tube is separated into two compartments by a porous barrier. The lower compartment contains the blue colored separation medium.
The upper compartment accommodates up to 30 mL of the blood specimen to be investigated. During a 20 minute centrifugation step the cells will be separated according to their different buoyant densities. The denser blood components such as erythrocytes and leucocytes migrate through the porous barrier into the lower compartment, thereby forcing the separation medium into the upper compartment. The less dense cell fraction, including the circulating tumor cells, will be enriched at the

interphase layer formed between the plasma and the separation medium in the upper compartment. After a harvesting and washing step the tumor cells are ready for further processing.

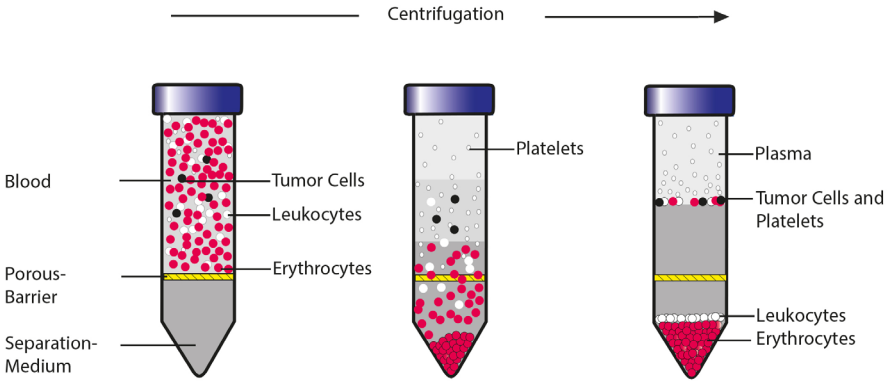

Fig. 1: **OncoQuick®** tubes: filled with whole blood before, during and after separation.

4/ REAGENTS, SUPPLIES, AND EQUIPMENT:

4.1 Reagents and materials provided with the kit

- 10 x OncoQuick centrifugation tubes
- To perform 10 separations of 15 mL – 30 mL of whole blood each.

4.2 Supplies and equipment not provided

- 50 mL polypropylene centrifugation tubes
- Centrifuge must be capable of generating at least 1600 relative centrifugation force (RCF) at 4°C with swinging bucket rotor and tube carriers/adapters for 30 x 115 mm tube size
- Disposable serological pipettes
- For the preparation of the washing buffer PBS + 0.5% (w/v) BSA the following reagents are required: NaCl, KCl, Na2HPO4 x H2O, KH2PO4, BSA (bovine serumalbumin), distillied H2O, a graduated cylinder and a bottle top filter, 0.22 µm (see 6.2. Preparation of washing buffer)
- Gloves appropriate for the protection against bloodborne infections (e.g. HBV, HCV or HIV)

5/ VALIDATION RESULTS:

In spiking experiments **OncoQuick®** has been extensively validated to determine the parameters defining the quality of the tumor cell enrichment. In these experiments 7 to 180 cultured and fluorescently labeled tumor cells from human breast (T47D and MDMB435s), lung (A549), and colon (COLO678) were spiked into 20 mL of whole blood from healthy donors that were stored for 48 hours at 2 – 8ºC prior to spiking. After enrichment the recovered labeled tumor cells were quantified. By utilizing this model system a linear (r2 > 0.99) average tumor cell recovery of 72% and an enrichment factor of 3.8 log was obtained. The variation of recovery for the spiked breast cancer cell line T47D was 8% in five different blood donors (see Tab. 1). Investigations of various high-risk cancer patients confirmed the spiking data and showed circulating tumor cells detected by immunocytochemistry (ICC).

Fig. 1: Validation results

Validation Parameter	Average	SD*	Range*
Recovery*	72%	18%	56% - 92%
Linearity*	0.994	0.002	0.991 - 0.995
Detection Limit*	1.46	0.002	1.08 - 1.83
Repeatability*	83%	8%	69% - 91%
Log Enrichment Factor*	3.8	0.08	3.72 - 3.88

***Footnotes**

- **Recovery:** Number of recovered tumor cells expressed in percent of the total number of spiked tumor cells.
- **Linearity:** Expressed as the regression coefficient r2 of the linear regression of the recovery.
- **Detection Limit:** Number of tumor cells required in 20 mL of whole blood in order to detect one tumor cell after separation.
- **Repeatability:** Variation of the recovered T47D mammary tumor cells obtained with five different lood donors performed on five different days expressed as SD.
- **Log Enrichment Factor:** Base 10 logarithm of the ratio of recovered tumor cells to remaining blood cells in collected fraction divided by the ratio of spiked tumor cells to blood cells in whole blood.
- **SD:** Standard Deviation
- **Range:** Highest and lowest measured value

6/ ENRICHMENT PROTOCOL

Blood specimens should be collected only by skilled individuals such as certified phlebotomists or physicians and according to the approved guidelines by the National Committee for Clinical Laboratory Standards (NCCLS)

6.1 Necessary preparations before starting the enrichment procedure

- Pre-cool the centrifuge to 4°C
- Prepare a bucket filled with ice
- Pre-cool **OncoQuick®** Tubes and blood specimen 10 – 15 min on ice
- Prepare the washing buffer (see section 6.2) with sterile water or with subsequent sterile-filtration
- Ensure the separation medium (blue) is completely in the lower compartment. If not, spin the **OncoQuick®** tube for a few seconds to bring the separation medium back into the lower compartment

6.2 Preparation of washing buffer

To perform 10 OncoQuick enrichments the preparation of 1000 mL of the washing buffer PBS + 0.5% (w/v) BSA are recommended as described: 0,27 M NaCl, 0,005 M KCl, 0,015 M Na2HPO4 x H2O, 0,003 M KH2PO4, 5 g bovine serumalbumin (BSA), add distilled H2O to a total volume of 950 mL and dissolve the reagents, adjust the solution to pH 7,4, add distilled H2O to a final buffer volume of 1000 mL. Check again pH and adjust to 7,4 if necessary. Subsequently, pass the washing buffer through a 0.22 µm bottle top filter. To avoid precipitation store the washing buffer at room temperature.

6.3 Enrichment Procedure

Important: Perform steps 2 and 3 at 2 – 8°C.

Washing steps can be performed at room temperature if more convenient. Also, please read Important Remarks (6.4) before performing this procedure.

1. Incubate **OncoQuick®** tube and blood specimen for 10 - 15 min on ice.
2. Fill the cooled whole blood (15 - 30 mL) gently into the upper compartment without disturbing the separation medium underneath the porous barrier.
3. Spin blood-filled OncoQuick® tube at 1600 x g and 4°C for 20 min in a swing bucket rotor with slow acceleration and no brake.

Warning: Excessive centrifuge speed (over 3200 RCF) may cause tube breakage, exposure to blood, and possible injury.

4. After centrifugation, the tumor cells will be in the interphase between the upper plasma (yellow/brownish) and the lower separation medium (blue). Usually this cell fraction is not visible.

- If platelet contamination does not affect subsequent detection procedures, the entire liquid volume above the porous barrier can be collected with a sterile serological pipette and transferred into a fresh centrifugation tube (alternatively, the entire liquid volume above the porous barrier can be harvested and transferred into a fresh centrifugation tube by means of decanting; please make sure that the blood cell pellet below the porous barrier is not disturbed during decantation). Subsequently, carefully rinse the inner tube wall and the surface of the porous barrier of the afore emptied OncoQuick®-tube with approximately 5 mL washing buffer to collect cells eventually adhering to these surfaces. Add them to the centrifuge tube already containing the transferred liquid volume and bring the volume to a total 50 mL using washing buffer. Mix the suspension by gently inverting the tube 5 times.
- If platelet contamination does affect subsequent detection procedures, discard the yellow-brownish plasma fraction using a sterile serological pipette leaving approximately 2.5 – 3 mL (layer thickness 0.4 – 0.5 mm) above the interphase. After that, the entire remaining liquid volume above the porous barrier can be collected with a sterile serological pipette and transferred into a fresh centrifugation tube (alternatively, the entire liquid volume above the porous barrier can be harvested and transferred into a fresh centrifugation tube by means of decanting; please make sure that the blood cell pellet below the porous barrier is not disturbed during decantation). Subsequently, carefully rinse the inner tube wall and the surface of the porous barrier of the afore emptied OncoQuick®-tube with approximately 5 mL washing buffer to collect cells eventually adhering to these surfaces. Add them to the centrifuge tube already containing the transferred liquid volume and bring the volume to a total 50 mL using washing buffer. Mix the suspension by gently inverting the tube 5 times.

5. Pellet the cells at 200 x g for 10 min.
6. Gently aspirate about 45 mL of supernatant without disturbing the cell pellet and leave the pellet in the remaining 5 mL of washing buffer. Resuspend the cells by gently vortexing or tapping the tube.
7. Add washing buffer to bring the volume to 50 mL. Cap tube and mix cells by gently inverting tube 5 times.
8. Pellet the cells at 200 x g for 10 min.
9. Aspirate as much supernatant as necessary without disturbing the cell pellet. Resuspend cell pellet in the desired medium for subsequent procedure.

6.4 Important Remarks

Remarks respective to single steps in the enrichment procedure:

Step 2
Do not introduce the blood right onto the porous barrier since this may result in mixing-up the separation medium with blood decreasing the separation quality. Slightly incline the OncoQuick®-tube and introduce the blood slowly down the side of the tube.

Step 3
For slow acceleration, the rotor should reach 1600 RCF in approximately 170 sec.

Step 4
To minimize further possible loss of tumor cells due to unspecific adsorption to dry plastic surfaces, pre-wet all dry surfaces with the washing buffer by simply pipetting up and down once or rinsing once to pre-wet all fresh serological pipettes or 50 mL tubes, respectively.

Step 6
In case of an unacceptably high contamination of erythrocytes, lysing of erythrocytes may be performed by the following procedure: After pelleting the cells (step 6), gently aspirate as much supernatant as possible without disturbing the cell pellet. Resuspend the cells by gently vortexing or tapping the tube. Add 5 mL erythrocyte lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, adjust with 1N NaOH to pH = 7.3) and incubate for 5 min at room temperature. Proceed with step 7 of the enrichment procedure.

7/ LIMITATIONS

7.1 Volume of Blood
15 mL to 30 mL of whole anticoagulated blood is the optimal volume for tumor cell enrichment. However, hematological parameters such as an abnormal hematokrit may affect the quality of separation. In this case the blood volume needs to be appropriately adjusted. Buffy coat specimen should be diluted at least 1:1 with PBS. To ensure an optimal separation quality, leave the blood or diluted buffy coat stand for about 10 - 20 min at 2 - 8°C to estimate the corpuscular volume. The total corpuscular volume should be within 5 - 15 mL to ensure optimal tumor cell enrichment.

7.2 Time

OncoQuick® yields best results if the blood specimen will be processed within 2 hours after collection. However, whole blood stored at 2 - 8°C for 24 - 48 hours still yields good results. Due to the added anticoagulant the cells tend to swell during extended storage times. This results in a higher degree of erythrocyte and leukocyte contamination in the tumor cell fraction. The dependence of blood cell contamination and storage time and temperature is illustrated in fig. 2.

7.3 Temperature of blood sample and OncoQuick®-tube

Ambient temperature of the blood sample and **OncoQuick®**-tube during steps 2 and 3 of the enrichment procedure must be maintained between 2°C to 8°C. At this temperature the separation medium is optimized to simultaneously enrich circulating tumor cells and to deplete most of the unwanted blood cells in the sample. Furthermore, enzymatic activities (e.g. proteases or RNases) are significantly decreased at low temperatures. This may increase the sensitivity of certain subsequent detection methods such as ICC or RT-PCR.

Fig. 2a:

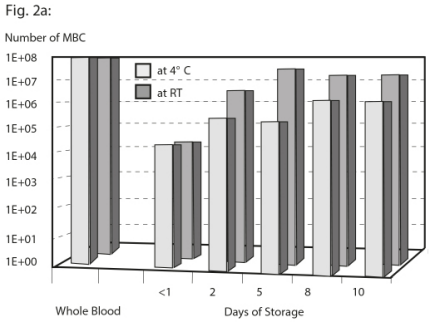

Fig. 2b:

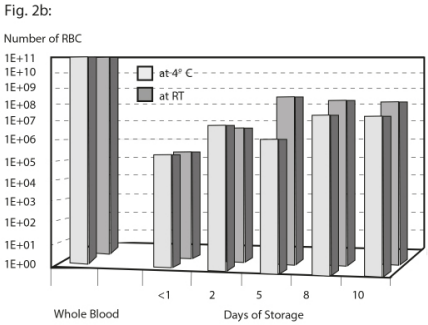

Fig. 2 : Number of contaminating mononuclear cells (a) and red blood cells (b) in respect to storage time and temperature after OncoQuick® separation.

7.4 Centrifugation

The RCF should be controlled at 1600 x g. The time of centrifugation should be 20 min. Longer centrifugation times usually do not increase the quality of separation.

7.5 Storage

Store **OncoQuick®** tubes upright at **4 – 22°C**. Under these conditions the expiry date for **OncoQuick®** tubes is 24 months from production.

The washing buffer should be stored at **room temperature** to avoid precipitation. Non sterile washing buffer should be stored at 4°C. If precipitates are visible in the washing buffer warm up the buffer to dissolve the precipitates and/or filter the buffer through a 0.22 µm filter (only necessary for subsequent ICC).

7.6 Contamination

Microbial contamination of reagents may alter the quality of separation.

8/ TROUBLESHOOTING

Problem	Possible Cause	Solution
Low tumor cell recovery	Temperature of separation medium and/or centrifuge above 8°C	Adjust the temperature to 4° C: prior to centrifugation keep **OncoQuick®** tube 10-15 min on ice and pre-cool the centrifuge to 4°C
	Tumor cell loss during collection of interphase cells	See remark to step 4 of section 6.4
	Tumor cell loss during washing steps	See remark to step 6 of section 6.4
	Red blood cell lysis too long	Shorten incubation time to a maximum of 5 min (see remark to step 6 of section 6.4)
Platelet excess	Washing step after separation omitted	Wash separated cells for 10 min at 200 x g (see section 6.3, step 7+8)
	High platelet count in whole blood	Wash separated cells additionally 1-2 x for 10 min at 200 x g (see section 6.3 step 9 +10)
No distinct cell layer visible	Few or no tumor cells in blood specimen	See section 6.3, step 4 and remark to step 4 in section 6.4

Problem	Possible Cause	Solution
No defined interphase between plasma (yellow/brownish) and separation medium (blue) visible	Acceleration speed too high	Extend acceleration time until rotor reaches final rpm (1600 x g) to approx. 170 sec
	Deceleration time too short	Turn off brakes of the centrifuge
	Incorrect size of adapter	Use 30 x 115 mm tube adapter
	Centrifuge not calibrated correctly	Have centrifuge calibrated
	Centrifuge speed too low	Increase speed to 1600 x g
	Centrifuge time too short	Increase time to 20 min
	Separation medium not completely in lower compartment prior to centrifugation	Spin separation medium into lower compartment prior to using **OncoQuick®**
	Total corpuscular volume too low	Increase blood volume to achieve a minimum total corpuscular volume of 5 mL (see section 7.1)
Red blood cell contamination	High red blood cell count (e.g. erythrocytosis); total corpuscular volume exceeds 15 mL	Reduce whole blood volume (see section 7.1)
	Blood storage time to long or stored at temperatures above 8°C	Perform separation step as soon as possible after collection, preferably within 2 hours (see section 7.2)
White blood cell contamination	Blood storage time too long or stored at temperatures above 8°C	Perform separation step as soon as possible after collection, preferably within 2 hours (see section 7.2)

9/ REFERENCES

- Robert Phelps, Edward McClay, Günther Knebel, Michael Dahm: Telomerase - A Suitable Marker for Circulating Tumor Cells in Peripheral Blood of Melanoma Patients. Poster presented at ASCB 2000, San Francisco (USA).
- National Committee for Clinical Laboratory Standards (NCCLS): Procedure for the Collection of Diagnostic Blood Specimens by Venipuncture; 4th ed., Approved Standard, H3-A4, National Committee for Clinical Laboratory Standards (NCCLS), Villanova, PA, 1998.
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- National Committee for Clinical Laboratory Standards (NCCLS): Protection of Laboratory Workers from Instrument Biohazards and Infectious Disease Transmitted by Blood, Body Fluids, and Tissue; Approved Guideline, M29-A, National Committee for Clinical Laboratory Standards (NCCLS), Villanova, PA, 1997.
- The Occupational Safety & Health Administration (OSHA): Final Standard for ccupational Exposure to Bloodborne Pathogens. 56 Fed. Reg. 64 175, Dec. 6, 1991; 29 CFR Part 1910.1030.
- Centers for Disease Control (CDC): Update: Universal Precautions for Prevention of Transmission of Human Immunodeficiency Virus, Hepatitis B Virus, and Other Bloodborne Pathogens in Health-Care Settings. MMWR 1988, 37 No. 24, p. 380.

10/ WARNINGS AND PRECAUTIONS

- Do not re-use OncoQuick tubes
- Do not use OncoQuick tubes after expiration date printed on the package label
- Do not use OncoQuick tubes if the clear blue separation medium becomes discolored or forms precipitates
- Do not use OncoQuick tubes for collection of material to be introduced into patients
- Excessive centrifugation speed (over 3200 RCF) may cause tube breakage, exposure to blood, and possible injury

11/ DISCLAIMER

This material is for research use only and is not intended for the use in humans or in-vitro diagnostic applications. No license for any such use is expressed or implied.

Caution:

- Handle all biological samples and blood collection lancets, needles, and blood collection sets ("sharps") in accordance with the policies and procedures of your facility
- Obtain appropriate medical attention in the event of any exposure to biological samples (e.g. puncture injury) since the samples may transmit HBV, HCV (hepatitis), HIV (AIDS), or other infectious diseases

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