# Biobanking – the First Step to Successful Liquid Biopsy Experiments

## Biobankování – první krok k úspěšným tekutým biopsiím

Almasi M.[1], Sevcikova S.[2], Penka M.[1], Krejci M.[3], Adam Z.[3], Vrublova P.[4], Jelinek T.[4], Hajek R.[4]

[1] Department of Clinical Hematology, University Hospital Brno, Czech Republic
[2] Babak Myeloma Group, Department of Pathological Physiology, Faculty of Medicine, Masaryk University, Brno, Czech Republic
[3] Department of Internal Medicine – Hematooncology, University Hospital Brno, Czech Republic
[4] Department of Haematooncology, University Hospital Ostrava, Czech Republic

### Summary

***Background:*** Archiving of biological materials in biobanks is considered to be the initial crucial part of research activities. Most often, biobanks are founded for research purposes since they allow collection of sufficient material for analysis of new or testing of previously identified biomarkers. Biobanking needs to quickly react to current needs of researchers as well as clinicians, it is not a rigid system. Laboratory analyses of monoclonal gammopathies are based on separation of plasma cells from bone marrow of patients. A specific problem is usually a lack of tumor cell fraction, which is due to location of tumor cell in bone marrow in combination with low infiltration. One of the challenges in clinical research is the necessity of changes in biobanking for samples allowing detection of minimal residual disease in the bone marrow but also from peripheral blood by the so-called liquid biopsies. ***Aim:*** The aim of this review is to show the importance of archiving biological material in the Czech Republic and to show concrete examples of its usage in hematooncology. ***Conclusion:*** A general problem in solving many research questions is the availability of a critical amount of specimens for statistical analysis. Obtaining critical amount of specimens of biological material can be quickly archived by cooperation of biobanks sharing both methodological standards and informations about the availability of samples for research projects.

### Key words

archiving – biological material – informed consent – multiple myeloma – plasma cells

### Souhrn

***Východiska:*** Archivace biologického materiálu v biobankách je v současné době zásadním krokem vedoucím k úspěchu výzkumných aktivit. Biobanky jsou zřizovány většinou k výzkumným účelům, protože umožňují shromáždění dostatečného množství materiálu pro analýzy nových nebo otestování již identifikovaných biomarkerů. Biobankování musí reagovat vždy na aktuální potřeby výzkumníků i kliniků, nejde o rigidní systém. Laboratorní analýzy monoklonálních gamapatií jsou založeny na separaci plazmatických buněk z kostní dřeně nemocných. Specifickým problémem je zpravidla nedostatek nádorové frakce buněk, což je dáno umístěním nádorových buněk v kostní dřeni v kombinaci s nízkou infiltrací. Jednou z výzev klinického výzkumu vyžadující změny nastavení v biobance je detekce zbytkového nádorového onemocnění v kostní dřeni, ale i z periferní krve tzv. tekutými biopsiemi. ***Cíl:*** Cílem této práce je přiblížit stávající stav v České republice a konkrétní případy jeho využití v hematoonkologii. ***Závěr:*** Obecným problémem při řešení řady výzkumných otázek je dostupnost kritického množství vzorků pro statistickou analýzu. Získání kritického množství vzorků biologického materiálu může být rychleji dosaženo spoluprácí biobank sdílejících jak metodické standardy, tak informace o dostupnosti vzorků pro výzkumné projekty.

### Klíčová slova

archivace – biologický materiál – informovaný souhlas – mnohočetný myelom – plazmatické buňky

This work was supported by grant of Ministry of Health, Czech Republic – Conceptual development of research organization (FNBr, 65269705) and by MH CZ – DRO – FNOs/2017.

Tato práce byla podpořena MZ ČR – RVO (FNBr, 65269705) a MZ ČR – RVO – FNOs/2017.

The authors declare they have no potential conflicts of interest concerning drugs, products, or services used in the study.

Autoři deklarují, že v souvislosti s předmětem studie nemají žádné komerční zájmy.

The Editorial Board declares that the manuscript met the ICMJE recommendation for biomedical papers.

Redakční rada potvrzuje, že rukopis práce splnil ICMJE kritéria pro publikace zasílané do biomedicínských časopisů.

Ing. Martina Almasi, Ph.D.
Department of Clinical Hematology
University Hospital Brno
Jihlavska 20
625 00 Brno
Czech Republic
e-mail: almasi.martina@fnbrno.cz

Submitted/Obdrženo: 25. 6. 2017
Accepted/Přijato: 28. 6. 2017

doi: 10.14735/amko20172S9

## Introduction

Biobanks are well-organized resources containing biological specimens and related information available for scientific research [1]. Biobanking is the first key step to successful clinical research. In the research of monoclonal gammopathies (MG), separated pathological populations of plasma cells (PC), plasma of the bone marrow (BM), serum and plasma of peripheral blood (PB) are most commonly obtained. All methods used for processing of these materials need to be standardized, optimized and performed well. For successful research and quick application of its results into clinics, it is important to process thousands of samples of clinical material that need to be archived in a biobank and available for various analyses of research teams [2].

**Tab. 1. Share (%) of individual categories of archived material.**

| Archived material | Share (%) |
|---|---|
| serum and plasma of PB | 60 |
| plasma of BM | 15 |
| negative fraction $CD138^-$ | 10 |
| positive fraction $CD138^+$ | 5 |
| mononuclear cells (BM) | 5 |
| mononuclear cells (PB) | 5 |

PB – peripheral blood
BM – bone marrow

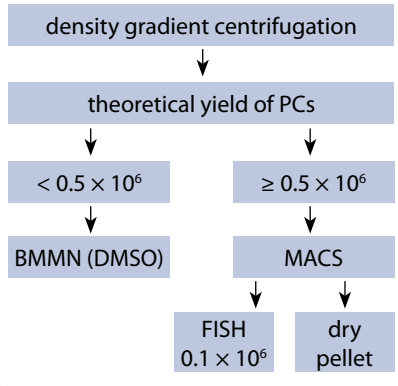



**Fig. 1. $CD138^+$ cells separation strategy.**

PCs – plasma cells, BMMC – bone marrow mononuclear cells, DMSO – dimethyl sulfoxide, MACS – magnetic-activated cell sorting, FISH – fluorescence *in situ* hybridization

## Processing and archiving of biological material

Since 1999, initial processing of biological materials and biobanking for research in the area of hematology, especially in monoclonal gammopathies, have been carried out at the Laboratory of Experimental Hematology and Cellular Immunotherapy at the Department of Clinical Hematology, University Hospital Brno (LEHABI OKH FN Brno). Currently, about 200 samples of BM are processed in our laboratory every year. The laboratory cooperates with the flow cytometry laboratory at the OKH FN Brno, which determines PC immunophenotype. LEHABI is focused on initial processing and archiving of biological material from PB (serum and plasma collection) and from BM (plasma of BM and immunomagnetic PC separation).

Since 2013, there is a new biobank in FN Ostrava that has been created based on the methods established in Brno. Besides the locality of Northern Moravia, it also collects samples of rare monoclonal gammopathies (amyloidosis, Waldenstrom macroblobulinemia) from the Vysegrad region. Since early 2017, both biobanks have been connected into a functional interface due to collaboration with the Institute of Biostatistics and analysis, Ltd. (IBA) using the electronic data collection system (EDC) and webdesign CLADE-IS (Clinical Data Warehousing Information System). Thanks to this new approach, available samples for a predefined research project may be found. The next step is connection to the Registry of Monoclonal Gammopathies that will help the research teams to set up research projects and will allow faster access to clinical data for publications. The list of samples are listed in Graph 1 and Tab. 1.

While BM biopsies are still the golden standard for diagnostics of MG, it is important to look for new markers available from PB that would decrease discomfort of the patients while giving the same or better information than BM biopsies. Our research team has been involved in the research of such molecules for quite some time [3–7].

## BM samples

In MG, most analyses are still based on separated PC from the BM of patients. There are several possible ways of separation, and our team has been working on this consistently [8–13]. To separate cells based on their different weight and volume, the BM sample is overlaid on density gradient (Ficoll, Histopaque). After centrifugation, a mix of monocytes and lymphocytes (the so-called mononuclear cells – MC) is separated. The principles of separation methods, which are followed for sample processing have been previously published [14]. Briefly, in BM mononuclear cells (BMMC) fraction, percentage of PC is measured by flow cytometry (using CD38 and CD138 markers). We have modified our previously published algorithm (Fig. 1) [14]. Instead of using infiltration of $CD38^+138^+$ cells in BMMC (> 5% for MACS separation – Magnetic-Activated Cell Sorting; < 5% for FACS – Fluorescence-Activated Cell Sorting), we are currently using a calculation of theoretical yield of $CD138^+$. Theoretical yield of PC $\times 10^6$ = % PC in BMMC/100× total number of cells in BMMC.

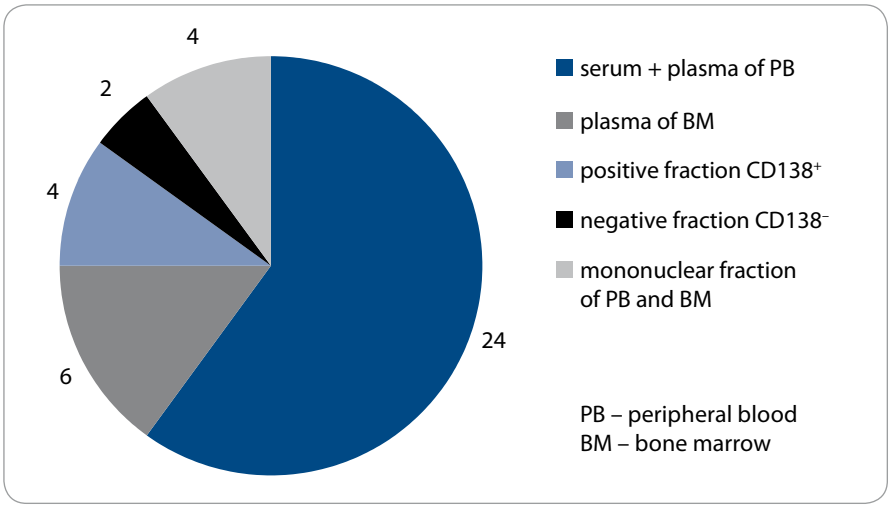
If calculation of theoretical yield is $\geq 0.5 \times 10^6$ separated $CD138^+$ cells, we use MACS separation, if it is lower, then BMMC are frozen at –80 °C in 1 mL of fetal bovine serum (FBS) containing 10% dimethyl sulfoxide (DMSO) in a freezing container. For long-term storage, these samples are transferred into liquid nitrogen containers and stored at –196 °C. These cells may be later used for sorting by FACS as previously described [14].

MACS separation is based on labeling the cells with monoclonal antibody coupled to magnetic particles. Target cells bind to the antibody and are captured in a separation column which is placed in a magnetic field in the autoMACS-Pro Separator (Miltenyi Biotec). Thus, the positive fraction contains PC while negative fraction (cells that did not bind the antibody) contains the rest of mononuclear cells.

Based on flowcytometry determination of infiltration of more than 10% of PC, the program Possels is used. For lower PC infiltration, Posseld2 program for double selection is used. In the next step, the column is removed from the magnetic field and enriched positive fraction (PC) is eluted. Then, yield and purity of the fractions

are determined. The number of BMMC before separation and the number of cells in the positive and negative fraction are calculated in the Burker chamber after staining with trypan blue and counted on inversion microscope. Purity of the positive fraction is measured by flowcytometry. At least in our hands, the median purity of PC separated fraction (using program Possels) is 91.3% (range 22.6–99.6%) as calculated on samples obtained between January 2013 and March 2017 (n = 197). Median purity of PC separated fraction (using program Posseld2) is 92% (range 2.2–99.7%) calculated for samples obtained in the same time interval as mentioned above (n = 151). In total, median purity of separated PC of samples archived in this time period is 92.3% (range 2.2–99.9%) (total number of samples n = 348).

Then, aliquots of separated BM cells (positive fraction as a dry pellet and negative fractions in DMSO) and aliquots of plasma of BM are stored.




**Graph 1. Number of archived aliquots of biological material for research purposes (in thousands).**

## Peripheral blood

Our attention is currently focused on liquid biopsies, meaning samples of PB. Both research teams in Ostrava and Brno have contributed to this Supplementum by several articles [15–17].

There are several pitfalls that can impact quality of samples and accuracy of further analyses. One of them is hemolysis of the specimen that may lead to release of cell material into serum or plasma. Therefore, it is important to note potential hemolysis for each sample so that these samples are not used for experiments that may be influenced by this fact.

Serum is the liquid portion of the blood without cells and clotting factors. The cells and clotting factors must be removed from the PB sample. Adequate time (30–60 min at room temperature) is required for the clot to form. Serum samples that are allowed to rest for less than 30 min are likely to retain cellular elements and other contaminants. On the other hand, samples that are allowed to rest for more than 60 min, are endangered by elevated lysis of cells in the clot [18].

On the other hand, plasma includes cellular material as well as clotting factors. Thus, plasma collection tubes contain different anticoagulants such as ethylenediaminetetraacetic acid (EDTA), sodium citrate and heparin. Each of these additives can impact the protein makeup of the sample in different ways. Based on our experience, heparin interferes with many kinds of analyses; therefore, we are currently using only EDTA collection tubes.

Recently, we have started separating circulating PC from PB. This cell fraction is found in very low quantities in PB and thus its analysis is a challenge. We use a two-step separation by MACS. In the first step, negative selection of non-PC is performed. Non-PC are indirectly magnetically labeled with a cocktail of biotin-conjugated monoclonal antibodies and anti-Biotin MicroBeads. Non-PC are retained in the magnetic field of a separation column. In the second step, $CD138^+$ PC are directly labeled with CD138 MicroBeads and isolated by positive selection from the previously enriched PC fraction. After the column is removed from the magnetic field, $CD138^+$ PC are eluted into the positive cell fraction. This depletion approach is faster and more dependable than FACS.

## Proper biobanking – aliquots and storage temperature

Biobanking is a fast developing field using standard operating procedures (SOP). Quality criteria are based on the conditions needed for research purposes, therefore there is not a universal standard for sample processing, and the needs of the research team may change in time. Thus, biobanking need to flexibly adapt to these changes. At the same time, there are known conditions that directly influence quality of archived material.

All samples should be immediately transported for processing. Long and improper handling of sampled BM and PB have detrimental effect on quality of cells as well as isolated nucleic acids. SOP for cell isolation should be used to ensure best quality samples for further analyses. All SOP need to be regularly evaluated and quality controls need to be performed.

Cryogenic preservation of cells is a major downfall for further analysis if improper procedures are used – incorrect cryogenic media, not standardized ways of freezing and thawing of samples all influence viability of the cells as well as quality of isolated nucleic acids [19].

Optimal long-term storage temperature is at least –80 °C. Samples of serum and plasma are of better quality if smaller volume aliquots are prepared so that each aliquot is thawed only once as freezing and thawing cycles have a dramatic negative impact on sample quality. Smaller volume aliquots simplify sharing samples and usage for various analyses. PB samples (serum, plasma, whole blood) are usually stored at –80 °C, in 0.5–1 mL aliquots.

On average, we archive 5–10 mL of serum, 3 mL of plasma from PB using

EDTA, 3 mL of plasma of BM. Separated PC are divided into several aliquots based on the total number of separated cells, so for most samples, more than 1 aliquot is archived.

## Separation of cells

For some biological analyses, separation of certain subpopulations are necessary (in the case of MG, these are malignant PC) that are found in the BM or PB only in a small portion. Thus, it is necessary to use a separation technique based on the amount and purity of the cell population, its percentages and presence of a specific marker necessary for the separation.

Every method has varying needs for amount of cells and their purity. For i-FISH (interphase fluorescence *in situ* hybridization), minimal purity is 70% and amount of separated $CD138^+$ cells fraction is $7 \times 10^4$. For other methods, optimal purity is above 90%.

For analysis using RNA, native cells are preferred as they allow for purification of high integrity RNA. Frozen samples may prove to be challenging for integrity and purity of isolated RNA and some changes in isolation procedures may be necessary. For successful isolation of RNA, the minimal amount of $CD138^+$ cells is usually $0.35 \times 10^6$ cells; on the other hand, for other analyses, dry pellets of $0.3–0.5 \times 10^6$ $CD138^+$ cells are used, frozen at liquid nitrogen at –196 °C. Generally, long-term storage of RNA is not recommended as RNA disintegrates even at lower temperature; thus, storage in RNA-stabilizing solutions or storage of cDNA is more appropriate.

While DNA is more stable, integrity and quality of DNA directly influences whole genome amplification [20] and may influence even detection of single nucleotide polymorphisms [21,22]. Repeated freeze-thaw samples are not recommended even for DNA.

## Informed consents and ethical issues of biobanking

Ethical issues surround usage of human biological material, most often it is the protection of personal information of patients and anonymized usage of samples. For research purposes, coding of samples and clinical data without identifying information are necessary. Each patient is given a unique code so that direct identification of the patient is impossible and only a limited number of medical staff may identify the patient. This is a pseudo-anonymous form of protection of the patient and his/her data as no other person is capable of identification of the patient.

All research projects need to be approved by an ethics committee of the hospital and all patients participating in any research project need to sign an informed consent form. All volunteering patients have to be informed about research purposes of the project and may be enrolled into the project only after the form is signed. Otherwise, the samples may not be used for any research purposes whatsoever.

## Conclusion

A key requirement for clinical research is sufficient amount of clinical material obtained from successful and high-quality initial processing of biological material. A general problem in solving many research questions is the availability of a critical amount of specimens for research projects to enable robust statistical analysis. That is why sample archiving and multicentric collaboration are key elements for many research analyses.

Presented biobanks in Brno and Ostrava provide sufficient amount of clinical material for research projects in multiple myeloma. At the same time, amount of archived material of rare diagnoses such as amyloidosis and Waldenstrom macroglobulinemia have been increasing thus creating a basis for further research in these rare and rarely studied diseases.

## References


**1.** Yuille M, van Ommen GJ, Bréchot C et al. Biobanking for Europe. Brief Bioinform 2008; 9(1): 14–24.

**2.** Hájek R, Almaši M, Říhová L et al. Pokrok v oblasti mnohočetného myelomu a vývoj souvisejícího laboratorního zázemí. Vnitr Lek 2012; 58(7–8): 135–140.

**3.** Sevcikova S, Kubiczkova L, Sedlarikova L et al. Serum miR-29a as a marker of multiple myeloma. Leuk Lymphoma 2013; 54(1): 189–191. doi: 10.3109/10428194.2012.704030.

**4.** Kubiczkova L, Kryukov F, Slaby O et al. Circulating serum microRNAs as novel diagnostic and prognostic biomarkers for multiple myeloma and monoclonal gammopathy of undetermined significance. Haematologica 2014; 99(3): 511–518. doi: 10.3324/haematol.2013.093500.

**5.** Kubiczkova-Besse L, Sedlarikova L, Kryukov F et al. Combination of serum microRNA-320a and microRNA--320b as a marker for Waldenström macroglobulinemia. Am J Hematol 2015; 90(3): E51–E52. doi: 10.1002/ajh.23910.

**6.** Besse L, Sedlarikova L, Kryukov F et al. Circulating Serum MicroRNA-130a as a Novel Putative Marker of Extramedullary Myeloma. PLoS One 2015; 10(9): e0137294. doi: 10.1371/journal.pone.0137294.

**7.** Sedlaříková L, Bešše L, Novosadová S et al. MicroRNAs in urine are not biomarkers of multiple myeloma. J Negat Results Biomed 2015; 14: 16. doi: 10.1186/s12952-015-0035-7.

**8.** Fišerová A, Hájek R, Doubek M et al. Imunomagnetická separace myelomových buněk. Klin Onkol 2001; 14(2): 46–50.

**9.** Čumová J, Burešová I, Kovářová L et al. Selekce plazmatických buněk. Klin Onkol 2008; 21 (Suppl): 190–194.

**10.** Cumova J, Kovarova L, Potacova A et al. Optimization of immunomagnetic selection of myeloma cells from bone marrow using magnetic activated cell sorting. Int J Hematol 2010; 92(2): 314–319. doi: 10.1007/s12185-010-0651-4.

**11.** Burešová I, Čumová J, Kovářová L et al. Srovnání selekce plazmatických buněk metodami MACS a FACS. Klin Onkol 2008; 21 (Suppl): 195–197.

**12.** Buresova I, Cumova J, Kovarova L et al. Bone marrow plasma cell separation – validation of separation algorithm. Clin Chem Lab Med 2012; 50(6): 1139–1140. doi: 10.1515/cclm-2012-8837.

**13.** Burešová I, Kyjovská D, Kovářová L et al. Algoritmus separace plazmatických buněk ze vzorků kostní dřeně. Klin Onkol 2011; 24(1): 35–40.

**14.** Potáčová A, Štossová J, Burešová I et al. Sample Processing and Methodological Pitfalls in Multiple Myeloma Research. Klin Oncol 2011; 24 (Suppl): 18–24.

**15.** Kubaczkova V, Sedlarikova L, Bollova B et al. Liquid Biopsies – the Clinics and the Molecules. Klin Onkol 2017; 30 (Suppl 2): 2S13–2S20.

**16.** Bezdekova R, Penka M, Hajek R et al. Circulating Plasma Cells in Monoclonal Gammopathies. Klin Onkol 2017; 30 (Suppl 2): 2S29–2S34.

**17.** Kufova Z, Sevcikova T, Growkova K et al. Biomarkers in Immunoglobulin Light Chain Amyloidosis. Klin Onkol 2017; 30 (Suppl 2): 2S60–2S67.

**18.** Tuck MK, Chan DW, Chia D et al. Standard operating procedures for serum and plasma collection: early detection research network consensus statement standard operating procedure integration working group. J Proteome Res 2009; 8(1): 113–117. doi: 10.1021/pr800545q.

**19.** Almáši M, Hájek R. Banky biologického materiálu. In: Slabý O et al (eds). Molekulární medicína. 1. vyd. Praha: Galén 2015: 42–44.

**20.** Ševčíková T, Growková K, Kufová Z et al. Biobanking strategy and sample preprocessing for integrative research in monoclonal gammopathies. J Clin Pathol 2017. pii: jclinpath-2017-204329. doi: 10.1136/jclinpath-2017-204329.

**21.** Almasi M, Sevcikova S, Svachova H et al. Polymorphisms Contribution to the Determination of Significant Risk of Specific Toxicities in Multiple Myeloma. Klin Onkol 2011; 24 (Suppl): S39–S42.

**22.** Almasi M, Sevcikova S, Slaby O et al. Association study of selected genetic polymorphisms and occurrence of venous thromboembolism in patients with multiple myeloma who were treated with thalidomide. Clin Lymphoma Myeloma Leuk 2011; 11(5): 414–420. doi: 10.1016/j.clml.2011.03.024.