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FLUORESCENT AND PEPTIDE CHARACTERISTICS OF THE HUMAN PROBIOTIC BACTERIA CULTURES

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ABSTRACT

A highly sensitive rapid analysis of the intrinsic fluorescence of culture supernatants, their ultrafiltrates and concentrates of proteins and exopolymer substances of *Gram* positive bacteria on a hydrophobic porous membrane is proposed using the example of probiotic strains of lactobacilli and bifidobacteria and lactobacillar multiprobiotic *Acylact*. The method was confirmed by analysis using a fluorescent dye *SYPRO protein blot stain*. In addition, an approach to the determination of partially hydrolyzed protein in fractions of cultures of *Gram* positive bacteria is described. A method of highly sensitive comparative (ranking of a parameter in sequences) microanalysis of partially hydrolyzed protein in culture supernatants is proposed using the example of lactobacilli probiotic strains and their consortium *Acylact*. *Acylact* formulas have been calculated, taking into account the contribution of ingredient strains, in the form of coupled ordered sequences of strains and *Acylact* - all ranked by the content of cultural peptides and proteins.

KEYWORDS: lactobacilli, bifidobacteria, multistrain consortium, *Acylact*, cultures, peptides, proteins, ranking, fluorophores, solid-phase assay, live image.

ABBREVIATIONS

BSA bovine serum albumin
CF culture fluids
Cn concentrates
FBC fluorophores-binding complexes

FCM fluorophores-containing molecules
FSB phosphate-salt buffer pH 7.4
PHP partially hydrolyzed proteins
PSB phosphate-salt buffer pH 7.2-7.4
Sp supernatants
SPA solid-phase analysis

1. INTRODUCTION

Culture fluids (CF) are sources of fluorogenic biologically active low and high molecular weight substances. Low molecular weight substances are represented by amino acids, [glyco]peptides and others that are capable of detecting fluorescence excitation, characterizing products in CF and facilitating monitoring of their formation and consumption. High molecular weight substances are represented by proteins, protein–containing complexes and others with varying degrees of masked fluorophores and/or interacting with fluorescent metal cationic dyes. Such fluorophore-containing molecules (FCM) and fluorophore-binding complexes (FBC) are involved in signaling, synergistic/coordinated cascading processes of nano/micro- and macrosystem bio-recognition as selective recognition, energy transfer and initiation of cascade reactions, perform vital macro functions in the environment and on the surface of bacteria.

CF are also sources of physiologically active partially hydrolyzed protein (PHP). Spectrophotometric analysis methods are highly sensitive and simple. In spite of this, testing PHP using different methods leads to a significant variation in the results.

The aim is to use solid-phase analysis (SPA) in a live image to investigate the types of FCM and FBC in CF of *Gram* positive bacteria using the example of probiotic strains of human bifidobacteria and lactobacilli, as well as of *Acylact*, a multi-strain consortium. In addition, the aim is also to optimize the determination of PHP in CF of *Gram* positive bacteria using the example of lactobacilli, and calculate the ranked peptide formulas of the *Acylact* and the its ingredient strains.

2. MATERIALS AND METHODS

CF samples were used from bacteria grown in casein yeast medium with 5 salts (CY-5s) during 18-24 hours at 37^{o}C,_with rotational stirring. Protein samples and supernatants (Sp) were studied after freezing (upper formation of a layer of plums that are removed) and

thawing as well as after pasteurization (30 minutes of boiling). The samples included sterile Sp (after membrane microfiltration through patron *Steriflip*, Millipore); and high molecular mass Cn (> 27 kD; the concentration factor – 40-60 times; obtained using membrane ultrafiltration through *Centricon Plus-20*, Millipore) of *Lactobacillus helveticus* (strains NK1 and 100ash), *L. casei* K3III24, multiprobiotic *Acylact* (NK1+100ашш +K3III24), and *Bifidobacterium longum* MS-42.

The samples of preparations were diluted in 10 mM phosphate-salt buffer 7.4 (PSB) of fat-free and demulsified supernatants of CF, their high-molecular concentrates (Cn) (more than 27 kD; obtained in *Centricon Plus-20* centrifuge patrons, Millipore) and ultrafiltrates as supernatants (Sp) (obtained after *Centricon Plus-20* centrifuge patrons) of CF of probiotic strains including *Lactobacillus helveticus* (strains NK1 and 100ash), *L. casei* K3III24, *Bifidobacterium longum* MS-42 and *Acylact*.

Protein was determined in the region of absorption of peptide bonds according to *Waddel*, 1956: [mcg/ml] = (D_{215} - D_{225}) x 144, where 144 is the coefficient calculated for bovine serum albumin (BSA) - non–glycosylated, free of lipids and protein impurities preparation, with high hydrophobicity), D – optical density at 215 and 225 nm. The linear dose dependence of D_{215} or D_{225} remains in the range from 0 to 5. Protein determination according to *Kalkar* (1947) was also used in the absorption regions of aromatic amino acids at 280 and 260 nm (Trp, Tyr) >> Phe: [mg/ml]= 1.45 x D_{280} - 0.74 x D_{260}. The color impurities in CF were evaluated by D_{420}, which directly correlated with the visually weakly pronounced color of lactobacillar Sp. Solutions of bovine serum albumin (BSA) and human IgG were used in the controls. All measurements are made in a quartz cuvette with an optical path length of 1 cm. The samples were stored in small portions in eppendorf plastic tubes at -35^{o}C, the samples were diluted in a phosphate-salt buffer pH 7.2-7.4 (FSB) before use.

To increase the sorption of the material in the dots on the hydrophobic membrane *Immobillon P* (Millipore) and to increase the number of exposed fluorophores, the membrane blots were treated with 2-5 mM dithiothreitol in PSB (80^{o}C, 6-7 min). To exclude the effect of hydrolases on SPA, CF samples were pasteurized by boilling for 30 min. Samples of CF contained up to 1 mcg/ml of hydrophobic protein. Such samples (3 mcl serial dilutions of CF [initially concentrated 60-100 times CF] or BSA [initially 2 mg/ml in PSB] were used in dilutions of 10; 100; 1000; 10,000 and 100,000 times; the initial stock dilutions were prepared in advance and stored in eppendorfs at -35^{o}C) were applied in rows to the centers of

squares/cells on a hydrophobic PVDF porous membrane *Immobillon-P* (Millipore). The fluorescence of the components (either on their own or after treatment with *SYPRO Ruby protein stain* (Bio-Rad, USA) [herein after referred to as SYPRO] - comparable to colloidal gold in sensitivity; based on *Ruthenium* cations [Ru^{+}]; does not react with nucleic acids, has excitation peaks at 280 and 450 nm and fluorescence emission peak at near 618 nm) was determined on dry blots in live image mode in the *BioChemi System* (UVP; excitation at 254 nm [excitation of amino acids such as Phe, Tyr and Trp, their modifications, as well as maximum energy excitation of SYPRO) or 365 nm (more specific excitation of SYPRO), and also registration of a series of luminescent patterns using a *Coomassie* light filter (excitation at 254 nm allows to evaluate the protein expression and its contribution to fluorescence in dilutions of CF supernatant or its concentrate before processing the blot using SYPRO) or an *Ethidium Bromide* light filter (transmission 570-640 nm as orange light); The interval of the step fluorescence accumulation time is 270 – 60,000 milliseconds (obtaining complementary information, depending on the optimization and the task type). In some cases, to increase the sensitivity, measurements were carried out under the conditions of blot thermo-stating at 50-55°C. To quantify the fluorescence, the pillbox regions were scanned. The *LabWorks4* software package was used in the analysis.

3. RESULTS AND DISCUSSION

3.1. Fluorescent components of CF of the human probiotic bacteria

3.1.1. FCM with intrinsic fluorescence of CF of the human probiotic bacteria

SPA revealed the presence of detectable unstable fluorescence (appearing at dilutions, as in the case of BSA, or capable of disrupting dose dependence in dilutions, as in the case of CF Sp Cn; dose dependence was not violated in the case of CF Sp) in the composition of sorped preparations (high-molecular Cn, as well as initial CF Sp; BSA was detected at a dilution of 100,000 times [20 picograms in the minimal on size of luminescence dot, Figure 1]), varying depending on the conditions of its excitation and accumulation, dilutions, and blot treatment type.

Two types of FCM in CF have been identified.

a) major – with a predominant contribution of (exopolymer substances)/ exopolysaccharides with low sorption capacity, with an isoelectric points in the near–neutral region;

b) minor - with good sorption in the zone of dot spot application, with dilutions of additives by 10-100 times.

The severity of FCM in lactobacilli was higher than in bifidobacteria, and the total content of FCM (excitation at 254 nm, *Ethidium Bromide* light filter, signal accumulation of 10,000-40,000 milliseconds) decreased in CF bacterial supernatants as the sequence 100ash > K3III24 > NK1 > MC42 > *Acylact*. In the case of excitation at 365 nm, the reproducibility of the results (convergence of measurements in pairs of different batches of CF samples of the same strain or consortium of strains) increased against the background of a decrease in fluorescence intensity (Figure 1).

At the same time, the ranking/ordering of FCM CF lactobacilli became more contrasting (differed more strongly) in the comparison groups: 100ash > K3III24 (group with elevated FCM levels); MS-42 > *Acylact* (group with relatively reduced FCM levels).

3.1.2. FBC in CF of CF of the human probiotic bacteria

The use of SYPRO in all cases improved, fully or partially restored the dose dependence of fluorescence in the series of BSA dilutions, Sp and their Cn. Since the intrinsic fluorescence of the dots was weak or absent during dilutions of the CF Sp by 100 times, further examination of the dots with high dilutions of the preparations was carried out using the fluorescent dye SYPRO, which increases the sensitivity of SPA, and makes it possible to register FBC in high dilutions of samples without the contribution of intrinsic fluorescence. In the case of Sp Cn, instability (for example, in FCM in the series of dilutions of the Cn of NK1 strain) and an order of magnitude reduced sensitivity were eliminated after heat treatment of the blot in the presence of dithiothreitol (Figure 2). As a result, a stable dose dependence of dithiothreitol-denatured FCM in the series of dilutions was achieved, including for dilutions of 1000 times it was possible apparent ranking of relative FCM content [excitation at 254 nm, *Coomassie* light filter, exposure 270-750 msec]: 100ash > NK1 > K3III24 > *Acylact*. A comparison of CF strains and consortium sequences ranked by fluorescence intensity indicates pronounced differences between high-molecular and low-molecular fluorophore components of Cn and Sp.

The data obtained indicate that the fluorophores in FBC are masked (i.e., FCM are found in complexes and supramolecular ensembles) in CF Sp and CF Sp Cn. As dilutions occur, unmasking and, as a result, refolding of the exposed structures of molecules occur. The native state of the molecules approaches folding, and, as a result, the appearance/manifestation of the fluorescent properties of FBC. Such high dilutions correspond to optimal working dilutions that ensure maximum fluorescence while maintaining biological activity.

3.2. PHP of CF of the human probiotic bacteria

The *Waddel* method (215, 225 nm) was compared with the *Kalkar* method (260, 280 nm).

In the study of lactobacillar Sp and their Cn, the (260, 280 nm)-method gave overestimated results compared to the (215, 225 nm)-method due to the non-specific contribution of colored impurities (chromophores) and nucleic acids to the protein determination by *Kalkar* method. Lactobacillar Sp were characterized by the presence of colored impurities that underestimate protein levels at relatively low Sp dilutions.

The convergence of the PHP content values by the (215, 225 nm)-method with Cn of BSA and IgG, prepared by dry weight in solution procedure, significantly increased in areas of high protein dilutions (1-10 mcg/ml). The latter was due to the dissociation of polypeptide complexes and aggregates under high dilution conditions. With such dilutions, the contribution of color impurities to the determination of PHP was practically absent.

Determination of PHP in Sp of CF (with or without cream) before and after heat treatment of Sp (boiling for 30 min) revealed an underestimated PHP content (strong variation was observed) in the case of native Sp of *Acylac*t cultures.

The reasons for the release of additional protein after pasteurization of *Acylact* was investigated. We compared 4 series of Sp treatments:

1) “Defrosted Sp with cream without heat treatment”,
2) “Defrosted Sp without cream without heat treatment”,
3) “Defrosted Sp with cream with heat treatment”,
4) “Defrosted Sp without cream with heat treatment".

The PHP content was.

Series 1: 25.70 mg/ml (4 measurements); range 35.08 – 25.40 = 9.68 (minimal deviation). The peptide formula of *Acylact* (which takes into account the peptide formulas of the ingredient strains) was: K3IIII24 > 100ash > NK1 >> *Acylact*.

Series 2: 23.89 mg/ml (4 measurements); range 35.08 – 13.31 = 21.77 (maximal deviation). The peptide formula of *Acylact*: K3IIII24 > *Acylact* > 100ash > NK1.

Series 3: 24.34 mg/ml (4 measurements); range 33.26 – 21.17 = 12.09. Peptide formula of *Acylact*: *Acylact* > K3III24 > NK1, 100ash.

Series 4: 11.19 mg/ml (4 measurements); range 20.56 – 3.02 = 17.54. The peptide formula of *Acylact*: 100ash > NK1 > *Acylact* > K3III24.

The lactobacillar PHP content in Sp of CF in three series (No.No. 1-3, with similar PHP values) was 24.64 mg/ml (12 measurements).

CONCLUSIONS

a) Series 1-3 are characterized without significant losses;

b) PHP content decreases in a number of series: No. 1 (as close as possible to the native Sp series; maximum spread of results) > (No. 3 > No. 2) [the best series for reproducibility of results] >> No. 4 (series No. 4 is not suitable for sample preparation before determining PHP);

c) levels of PHP in Sp exceed the content of BSA-similar hydrophobic proteins in Sp by more than an order of magnitude (the protein content in Sp is up to 2 mg/ml according to our data of dot-block analysis).

It should be noted that the above peptide formulas of *Acylact* can be also considered as peptide formulas of any ingredient strain of *Acylact* due to coupled connections between probiotic bacteria in sequences.

The PHP content (mg/ml) in Sp of *Acylact* and its ingredient strains was calculated for series No.No. 1-3, as well as for all series No.No. 1-4:

Acylact: 1) 16.93, 2) 25.40, 3) 33.26, 4) 9.07; 25.20 (series 1-3); 21.16 (series 1-4).

NK1: 1) 25.40, 2) 13.31, 3) 21.17, 4) 12.10; 19.96 (series 1-3); 18.00 (series 1-4).

100ash: 1) 25.40, 2) 21.77, 3) 21.17, 4) 20.56; 22.78 (series 1-3); 22.22 (series 1-4).

K3III24: 1) 35.08, 2) 35.08, 3) 21.77, 4) 3.02; 30.64 (series 1-3); 23.74 (series 1-4).

PHP's ranking has been declining in the sequences:

a) for all series 1-4: K3IIII24 > *Acylact* > 100ash > NK1;

b) for series 1-3: K3IIII24 > 100ash > *Acylact* > NK1.

Thus, K3III24 was the dominant PHP contributor to the peptide formula of *Acylact*. The determination of PHP with absorption in the area of peptide bonds gave stable, well–reproducible rankings for K3IIII24, 100ash, and NK1 (there was a convergence in this feature of *Acylact* with NK1, the dominant contributor to the severity and diversity of lectin and adhesin systems in the multi-strain consortium). Sp of bifidobacteria were characterized by a lower PHP content compared to Sp of lactobacilli, that reflected increased levels of proteolytic activity in CF of lactobacilli (and, accordingly, increased levels of physiologically active [oligo]peptides available to the body). The relative independence of the PHP

mesurement from the conditions in all series was observed in the case of 100ash. The maximum variation in the series was observed in the case of K3IIII24, which manifested itself as an "unstable" strain, whose Sp(s) were characterized by irreversible gradual prolonged sedimentation.

In the case of *Acylact*, "additional" PHP was released as a result of boiling Sp with cream (cream protein yield was observed), and boiling without cream sharply reduced the detectable PHP. In dilute solutions, the cream maintained structures in Sp with PHP shielded in micelles (the formation of micelles with different proteins by their current fusion and/or their partial destruction were observed by us during isoelectrofocusing Cn in a polyacrylamide gel plate in the cationic region of the pH gradient), and after removing the cream in dilute solutions, the destruction of micelles and the release of PHP from them continued so that without boiling Sp with the cream removed, the maximum PHP content was achieved in the case of *Acylact*. Boiling Sp without the stabilizing effect of cream led to a significant decrease in PHP contents, similar to the most pronounced decrease in protein levels in the case of K3III24 (it may be promising to add *Acylact* cream or another ingredient strain cream to Sp of K3III24 as a stabilizer during storage of Sp of K3III24).

The Sp of pasteurized *Acylact* without cream was closest to the Sp of strain K3IIII24 in terms of the instability of PHP properties. As a result, this led to unstable storage of the 4th series of Sp of *Acylact*, especially in procedures using freeze-thaw cycles. The volume of cream (dark ring 1-3 mm thick in 20 ml of frozen Sp in a standard polystyrene tube with a graduated volume of 25 ml) of *Acylact* and its strains varied in the range of 0.35-0.70 ml. The rate of thawing of cream over ice during defrosting decreased in the following order: (100ash [the best emulsifier and the best foaming agent during vacuum filtration of Sp in a cartridge] > NK1) [sequence fragment coincides with the relative maximum pronounced color] > *Acylact* > K3III24 (poorly recorded).

The severity of the dark brown shade of cream decreased in the range: 100ash > NK1 (there is no discoloring oxidoreductase system in NK1; it is the dominant contributor of high-molecular exopolymer substances to *Acylact*) >> *Acylact* (the maximum effect of the discoloring activity of the oxidoreductase system of extracellular and intracellular nature; the maximum availability of this system as antimicrobial one) ≥ K3III24. The color of the Sp did not correlate with the severity of the cream.

The results indicate that a balanced multi-strain consortium of *Acylact* increases the stability of the ingredient strains (in cases of strains similar to the unstable strain K3IIII24 with constant sedimentation) due to the donation/contribution of stabilizers by other ingredient strains.

The PHP content (mg/ml) in Cn corresponded to the concentration factor of Sp and the determination of the content of BSA-like proteins in lactobacilli Sp (1-2 mg/ml according to dot-block analysis) and varied in the intervals (in parentheses – a decrease in additive PHP after boiling Cn dilutions for 30 minutes): NK1: 62.5-69.0 (27.5%), 100ash: 65.5-75.1 (30.6%), K3III24: 75.5-78.6 (15.1%), *Acylact*: 70.5-73.5 (8.98%). The decrease in PHP in a number of heat-treated Cn (K3IIII24, *Acylact* > 100ash > NK1) or native Cn (K3IIII24 > *Acylact*, 100ash > NK1) was confirmed by the results of the dot-block determination of PHP (K3IIII24 > *Acylact* > 100ash > NK1). Heat treatment reduced the PHP in the samples by 10-30%. At the same time, the precipitation formed in the case of K3III24 was irreversibly aggregated (did not dissolve in PSB), unlike reversible precipitation of *Acylact*. The Cn color intensity was significantly lower than the initial Sp had.

In general, our proposed minimal (additions are indicated in parentheses) microanalysis algorithm for determining PHP in a sample (0.1-1 mcl of Sp CF is sufficient) of freshly obtained Sp includes.

*freezing the sample and removing the top layer of cream (instead of freezing, boiling is possible for 15-30 minutes to destroy the emulsion state and inactivate enzymes);

*defrosting, centrifugation, sterilization microfiltration (Cn can be also obtained by ultrafiltration);

*dilution of Sp (or Cn) to a level of 1-10 mcg/ml;

*precise measurement of PHP by *Waddell* method;

*recalculation of the PHP content in Sp (or recalculation of protein in Sp according to Cn and factor dilution data).

4. CONCLUSION

The proposed optimized SPA without or using dithiothreitol for monitoring and characterizing the intrinsic fluorescence of native FCM in CF and their fractions is highly sensitive in the detection of protein and non-protein fluorophores (including masked and unmasked in FCM and their complexes), a highly reliable rapid microanalysis tool (SPA time using SYPRO – less than 3 hours in study of strain differences in comparison with a multi-

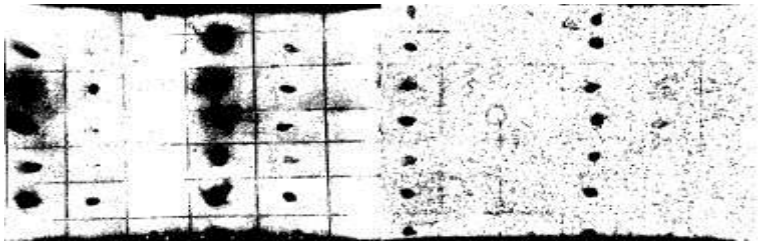
strain consortium) of CF bacterial centrifugates and filtrates, monitoring the processes of biosynthesis, assembling and hydrolysis of fluorescent signals in CF, evaluating the transfer of fluorescence energy to nearby environmental structures, and selecting strains for constructing probiotic consortia. The method is promising in the study of cultures of any *Gram* positive bacteria, including in variants of minicultures and individual cells, as well as for micropanel and biochip variants of SPA.

The use of FCM modification by SYPRO to confirm the results of intrinsic FCM fluorescence is not necessary under the conditions of using an established standardized procedure for registering the intrinsic fluorescence of known preparations (such as BSA). The determination of FCM and FBC by SPA is an important component of the overall laboratory analysis of the probiotic bacterial CF, including assessments of total and partially hydrolyzed protein, lectins, biosurfactants and other exopolymer compounds, the degree of emulsification, the degree of hydrolysis of components (including the presence of aromatic amino acids), the potential presence of glycated amino acids/peptides, the comparative low severity of pigmentation add-ons, availability of effective enzymatic hydrolytic and oxidoreductase systems (including those distributed in a strain-dependent manner in a consortium) – all aspects of complete analysis performed by us earlier.

The described PHP microanalysis algorithms are convenient for laboratory screening and other comparative studies of *Gram* positive bacterial cultures. The proposed microanalysis of peptide formulas of multi-strain consortia can be used in the design of multiprobiotics of the desired orientation, as well as in the study of microcultures and micro-samples of *Gram* positive bacteria.

Disclosure of conflict of interest

The authors declare no conflict of interest.



[Left blot] [Right blot]

Figure 1: The fluorescence of dot sample dilutions in the centers of squares/cells (the whole membrane as 6 x 7 cells).

On the left - the intrinsic fluorescence of the preparations.

On the right – after treatment with SYPRO (excitation at 365 nm, *Ethidium Bromide* light filter, exposure of 30,000 msec).

From left to right – dilution preparations (3 cells per preparation; BSA: 1000; 10,000; 100,000 times; CF supernatants: 10, 100, 1000).

From top to bottom – the preparations.

1st row: BSA (first 3 cells), BSA repeat (next 3 cells);

2nd row: *Acylact*, 100ash;

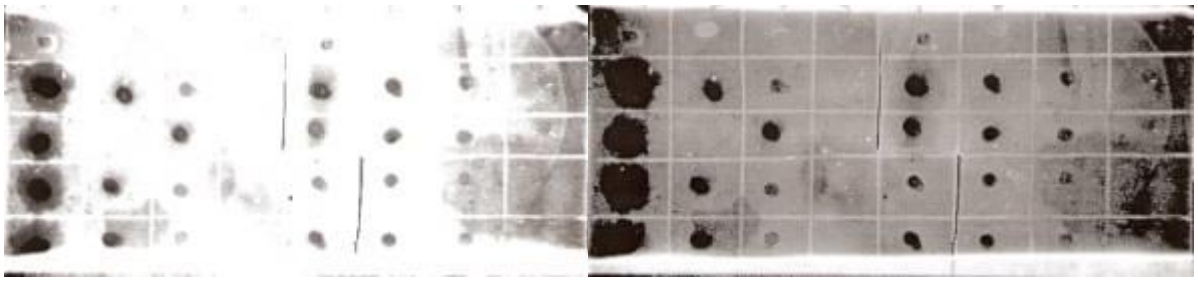
3rd row: NK1, K3III24;

4th row: MS-42, K3III24;

5th row: *Acylact*, MS-42;

6th row: NK1, 100ash;

7th row: BSA, MS-42.



[Left blot][Right blot]

Figure 2: Fluorescence of SYPRO-treated blots.

Left blot: without dithiothreitol treatment (left) or after dithiothreitol treatment (right: vertical borders are visible). Excitation at 254 nm, *Coomassie* light filter, exposure - 540 msec, 50°C. From left to right – dilutions of preparations (4 cells per preparation: 10; 100; 1000; 10,000).

Top-down – preparations.

1st row: BSA (4 cells), BSA repeat (next 4 cells);

2nd row: concentrates of 100ash (4 cells) and *Acylact* (next 4 cells);

3rd row: NK1 concentrate (4 cells, repeat – the next 4 cells);

4th row: K3III24 concentrate (4 cells), NK1 ultrafiltrate (after *Centricon Plus-20*) with more pronounced color impurities (4 cells: dilutions 10, 10, 100, 1000);

5th row: *Acylact* Cn of Sp of CF (4 cells), *Acylact* ultrafiltrate of Sp of CF with less pronounced colored impurities (4 cells: dilutions of 10, 10, 100, 1000).

Right Blot: like the left one, but in a different version (the fluorescence of the BSA quickly "discolors" under registration conditions at 50^{o}C).