SUPPORTING INFORMATION

Immobilization of biotinylated antibodies through streptavidin binding aptamer

D. Brambilla[*§], *L. Sola*[§], *F. Damin*[§], *A. Mussida*[§], *M. Chiari*[§]

**Corresponding author: dario.brambilla@scitec.cnr.it*

[§]National Research Council of Italy – Institute of Chemical Sciences and Technology (CNR – SCITEC), via Mario Bianco 9, 20131, Milan, Italy

1. Assembly of sensing elements on silica microbeads

As previously demonstrated by our group [1] silica micro particles coated with MCP-6 (an azide-containing polymer) can provide an effective immobilization of DBCO-modified biological probes, including ssDNA. Hence, we evaluated the possibility to immobilize StrepApt5 on the surface of beads, as well as the ability to perform reversible aptamer-mediated antibody immobilization.

1.1 Materials

Ammonium sulfate ($(NH_4)_2SO_4$), phosphate buffer saline tablets (PBS), Trizma base, 37% chloric acid (HCl), sodium phosphate (Na_3PO_4), sucrose monolaurate, sodium chloride (NaCl), ethanolamine, trehalose dehydrate, magnesium chloride ($MgCl_2$), NHS-PEG_4-biotin, Amicon Ultra 100MWCO centrifugal filters, streptavidin and polyclonal rabbit IgG were purchased from Sigma Aldrich (St. Louis, MO, USA). Bradford Protein Assay Dye Concentrate was purchased from Bio-Rad (Hercules, CA, USA). Goat antirabbit IgG was purchased from Jackson ImmunoResearch (Baltimore, PA, USA). Oligonucleotides were synthesized by MWG-Biotech AG (Ebevsberg, Germany): see Section 1.1.1 for oligonucleotide sequences. StrepApt5 was modified at 5' end with DBCO-linker, cDNA was used both unmodified and labeled with Cy5 at 5' end. Oligonucleotides were freeze-dried and resuspended in de-ionized water (DI water) at a final concentration of 100 µM before

use. StrepApt5 underwent a folding procedure before immobilization on solid supports: aptamer solutions were heated at 95°C for 8 min, transferred into ice at -20°C for 10 min and finally left at room temperature for 15 min.

Silica microbeads were purchased from Bangs Laboratories Inc (Fishers, IN, USA). Spectrofluorimetric analysis was performed using a Jasco FP-550 spectrofluorometer equipped with thermo-stated Peltier cell holder. Bradford protein assay were performed using a Thermo Labsystems Multiskan Ascent microplate spectrophotometer.

1.1.1 Oligonucleotide sequences

- *StrepApt5*: 5'-GGGAACGCACCGATCGCAGGTTTCCC-3'
- *cDNA*: 5'-ACCTGCGATCGGTGCGTTCCC-3'

1.1.2 Beads functionalization with StrepApt5

Silica microbeads (diameter = 1μm, 10% content in solids) were sonicated 10 min and stirred using a vortex shaker 30 sec in order to achieve optimal resuspension. 50 μL (= 5 mg) were transferred in a 1.5 ml tube and centrifuged to pellet beads (30 sec at 12,000 rpm). The same procedure was repeated after every washing or incubation step to remove supernatant from beads. Beads were washed twice with bidistilled water and then incubated with a MCP-6 solution (1% w/v) in 0.9 M ammonium sulfate for 1 h at 25°C under stirring. Beads were washed twice with bidistilled water and once with PBS added with 2 mM $MgCl_2$ (hereinafter referred to as PBS-M). Then, beads were resuspended in 100 μL of 10 μM DBCO-modified StrepApt5 in PBS-M (previously folded as described in Section 1.1) and incubated overnight at 37°C under stirring. Finally, beads were washed twice with PBS-M.

1.1.3 Assessing StrepApt5 immobilization efficiency on silica microbeads

5 mg of StrepApt5 decorated silica microbeads were resuspended in 100 μL of 10 μM Cy5-labelled cDNA in PBS and incubated for 1 h at 25 °C under stirring. After centrifugation the supernatant was recovered and the beads washed twice with 100 μL of PBS. After each washing step, the solution was centrifuged and the supernatant recovered. The supernatants were pooled and diluted 1:10 using PBS; 150 μL of the so obtained solution were added to 350 μL of PBS and the light emitted at 659 nm was determined by spectrofluorimetric analysis. The concentration of cDNA after incubation with beads was determined using a calibration curve. The amount of StrepApt5 bound to the beads was

assessed subtracting the concentration of cDNA before and after incubation with silica beads.

1.1.4 Streptavidin immobilization on StrepApt5 decorated silica microbeads

5 mg of StrepApt5 decorated silica microbeads were resuspended in 100 μL of 0.1 mg/mL (=10 μg) streptavidin in PBS-M and incubated 1 h at 25°C under stirring. After centrifugation the supernatant was recovered and the beads washed twice with 100 μL of PBS-M. After each washing step, the solution was centrifuged and the supernatant recovered. The supernatants were pooled and streptavidin concentration was measured by Bradford protein assay. As the negative control the same experimental procedure was repeated on 5 mg of MCP-6 coated silica microbeads.

1.1.5 Synthesis of biotinylated rabbit IgG

To 100 μL of 1 mg/mL solution of polyclonal rabbit IgG in PBS, 3.15 μL of 8.5 mM NHS-PEG_4-biotin (40 equivalents) were added and allowed to react for 30 min at 25°C. Reaction was quenched by adding 10 μL of 1 M Tris-HCl pH 8. After 5 min unreacted biotin linker was removed by centrifugation of Amicon Ultra 100MWCO centrifugal filter (3 × 5 min at 12.000×*g*). After centrifugation, the volume was adjusted to 100 μL with PBS.

1.1.6 Antibody immobilization on StrepApt5 decorated silica microbeads

5 mg of StrepApt5 decorated silica microbeads were resuspended in 300 μL of 1 mg/mL streptavidin in PBS-M and incubated 1 h at 25°C under stirring. Beads were washed twice with PBS-M and then incubated with 40 μL of 0.5 mg/mL (=20 μg) biotinylated rabbit IgG in PBS-M for 1 h at 25°C under stirring. After centrifugation the supernatant was recovered and the beads washed twice with 40 μL of PBS-M. After each washing step, the solution was centrifuged and the supernatant recovered. The supernatants were pooled and antibody concentration was measured by Bradford protein assay. As negative control the same experimental procedure was repeated on 5 mg of StrepApt5 decorated silica microbeads, incubated with native rabbit IgG without biotin modification.

1.2 Results & Discussion

Microarray experiments of aptamer-mediated antibody immobilization have demonstrated that the ability of StrepApt5 to immobilize streptavidin depends on its surface density. In particular, they highlighted the presence of a critical concentration of the aptamer that

provides the best immobilization rate for streptavidin. The optimal density is achieved by spotting the aptamer at 10 μM concentration. A similar dependence was expected on silica microbeads. Therefore, the concentration of DBCO-modified StrepApt5 to be used for microbeads functionalization was finely tuned.

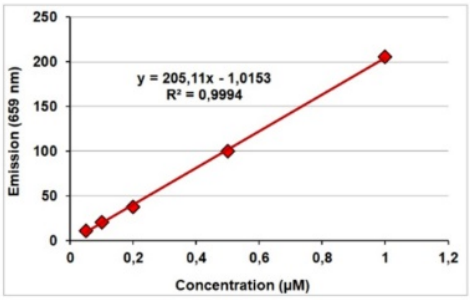
Previous experiments on DNA microarrays, performed using IRIS detection technique, revealed that by spotting ssDNA at a concentration of 10 μM, the density of the oligonucleotide on the surface ranges between 1-2 ng/mm^2 [2]. Knowing the density of DNA produced by incubating silica microbeads with different concentrations of DBCO-modified ssDNA [1], we extrapolated that the optimal amount of StrepApt5 immobilized on the surface could be obtained at 10 μM concentration also for beads functionalization.

In order to confirm this prediction, 5 mg of silica beads were coated using MCP-6 and functionalized with 10 μM StrepApt5 as described in Section 1.1.2. Functionalized beads were incubated with 100 μL of 10 μM Cy5-labeled cDNA, and the residual fluorescence in the supernatant was determined by spectrofluorimetry. The results, summarized in Figure S1, indicate that, under these experimental conditions, StrepApt5 is immobilized on the surface of silica microbeads with a density of 1.32 ng/mm^2. This density on the surface was considered to be optimal for further steps.

First of all, the ability of StrepApt5-functionalized beads to immobilize streptavidin was evaluated. To this scope, 5 mg of aptamer-functionalized beads were incubated with 100 μL of 0.1 mg/mL streptavidin in PBS-M and then washed as described in Section 1.1.4. As the negative control, 5 mg of MCP-6 coated silica microbeads were treated in the same way.

Finally, the supernatant was recollected and streptavidin concentration was assessed by Bradford protein assay. The results are shown in Figure S2. As it can be noticed, 145.3 pmoles are effectively captured on beads functionalized with StrepApt5, while only a negligible amount is measured on the negative control. These results confirmed that the aptamer, once immobilized on silica microbeads, retains its ability to effectively and selectively bind streptavidin.

a)



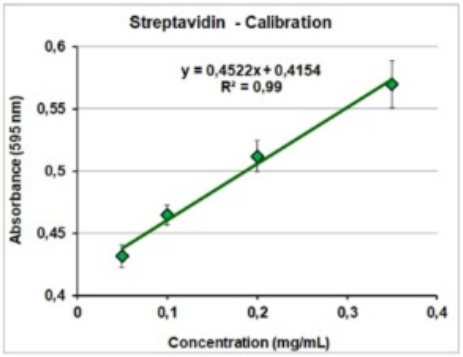
b)

Bound StrepApt5 (pmol)	Molecular Weight (g/mol)	Surface Area* (μm^2)	Surface Density (ng/mm^2)
117.08 ± 2	8452	$7.5*10^8$	1.32 ± 0.01

***Figure S1**. a) Calibration Curve for Cy5-labeled cDNA. b) Amount of StrepApt5 immobilized on beads expressed as pmoles and surface density.*

** Results are referred to 1 mg of silica microbeads.*

a)



b)

	StrepApt5 functionalized beads	Negative control
Immobilized streptavidin (pmol)	**29.06 ± 0.1**	6.1 ± 0.8

***Figure S2**. Results for streptavidin immobilization on StrepApt5-functionalized silica microbeads. a) Calibration Curve for streptavidin using Bradford protein assay. b) Amount of streptavidin captured on StreptApt5-exposing beads and on the negative control. Results are referred to 1 mg of silica microbeads.*

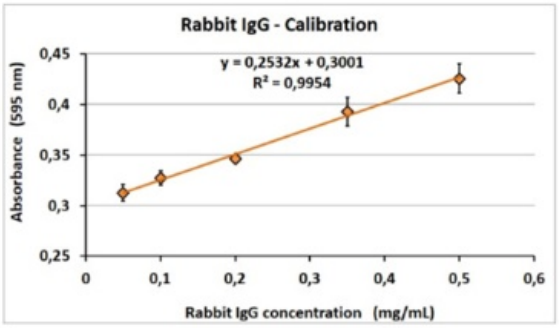
Once the feasibility of the aptamer-mediated immobilization of streptavidin has been demonstrated, we investigated the immobilization of biotin-labeled antibodies on the surface of aptamer-bound streptavidin.

To this purpose, polyclonal rabbit IgG was biotinylated according to Section 1.1.5. MCP-6 coated silica microbeads were functionalized with StrepApt5 and streptavidin as described in the Sections 1.1.2 and 1.1.4; 5 mg of these beads were incubated with 40 µL of 0.5 mg/mL

biotinylated rabbit IgG in PBS-M. As a negative control, 5 mg of beads were prepared in the same way and were incubated with unmodified rabbit IgG, which, lacking of biotin modification, was not supposed to bind to streptavidin.

After incubation the supernatants were recollected and the antibody concentration was assessed by Bradford protein assay. Subtracting the residual antibody concentration to the initial concentration, it was possible to determine the amount of antibody captured on the surface of beads under these experimental conditions. Results are shown in Figure S3 and clearly demonstrate that biotinylated IgGs are effectively captured on aptamer-bound streptavidin. This interaction is driven by the affinity between biotin and streptavidin, as the same antibody without biotin modification could not be immobilized on the surface of beads.

a)



b)

	Biotinylated rabbit IgG	Negative control
Captured antibody (µg)	3.46 ± 0.1	< 0

Figure S3*. Results for antibody immobilization on StrepApt5-functionalized silica microbeads. a) Calibration Curve for rabbit IgG using Bradford protein assay. b) Amount of rabbit IgG captured on beads functionalized with aptamer-bound streptavidin and on the negative control. Results are referred to 1 mg of silica microbeads.*

1.3 Conclusion

The results confirmed that StrepApt5 is able to bind streptavidin. Aptamer-mediated immobilization of streptavidin is then successfully exploited to anchor biotinylated antibodies on the surface. The total amount of biotinylated antibody immobilized through StrepAp5 (i.e. 3.46 µg/mg) is comparable with that obtained from commercially available beads (e.g. Dynabeads M-270 Streptavidin, where 1 mg of beads are declared to bind up to 10 µg).

2. Characterization of sEVs isolated from HEK-293 cell culture supernatants by ultracentrifugation

2.1 Nanoparticle Tracking Analysis: All samples were analyzed using Nanosight NS300 (Malvern Panalytical, Malvern, UK) configured with 532 nm laser. Videos were analyzed by the in-build NanoSight Software NTA 3.2 Dev Build 3.2.16. The Camera type, Camera level, and Detect Threshold were sCMOS, 11 and 4, respectively. A syringe pump with constant flow injection was used. The number of completed tracks in NTA measurements was 5 (a 60 seconds movie was registered for each measurement). All samples were diluted in PBS to a final volume of 1 mL. The ideal concentration was assessed by pre-testing the optimal particle per frame value (20-100 particles per frame).

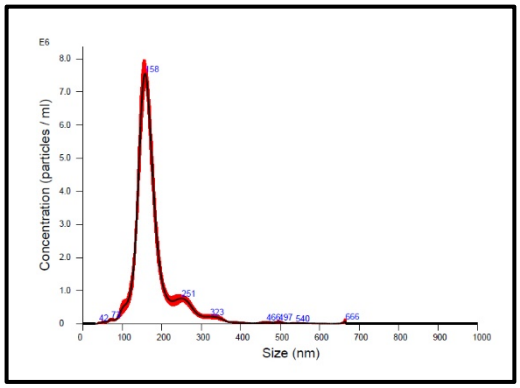


Figure S4*. Nanoparticle Tracking Analysis of EVs isolated from HEK-293 cell culture media by ultracentrifugation.*

2.2 Western Blot Analysis: Protein content was determined using protein assay kit (BioRad, CA, USA). Bovine serum albumin (BSA) was used as standard. For tetraspanin detection, sample was lysed in non reducing sample buffer (62.5 mM Tris–HCl pH 6.8, 10% glycerol, 2% SDS, and 0.04% bromophenol blue) and boiled for 5 min at 95°C. Then, 15 µg of proteins were loaded on 12% SDS-PAGE gel. For the detection of other proteins, sample was lysed in reducing sample buffer (62.5 mM Tris–HCl pH 6.8, 10% glycerol, 2% SDS, 1.25% 2-mercaptoethanol and 0.01% bromophenol blue) and boiled for 5 min at 95°C. Then, 24 µg of proteins were loaded on 12% SDS-PAGE gel. After protein separation, gels were electro-transferred onto a nitrocellulose membrane. Nonspecific binding sites were blocked with 5% (w/v) skimmed milk in T-TBS (150mM NaCl, 20mM Tris-HCl pH 7.4, and 0.5% Tween 20). Membranes were incubated overnight at 4°C with the following antibodies: anti-CD63 (1:1000, #556019, BD Pharmingen, CA, USA), anti-CD9 (1:1000, #555370, BD Pharmingen, CA, USA), anti-TSG101(1:800, #NB200-112, Novus Bio, CO, USA), anti-Alix (1:500, #sc-271975, Santa Cruz, CA, USA) and After several washes in T-TBS, membranes were incubated with goat anti-mouse IgG conjugated to horse-radish peroxidase (1:5000,

#170-6516, BioRad Laboratories Inc., CA, USA) for 45 min. Positive immunoreactive bands were detected by the enhanced chemiluminescence method (Immobilon™ HRP substrate, #WBKLS0500, Millipore Corp., MA, USA).

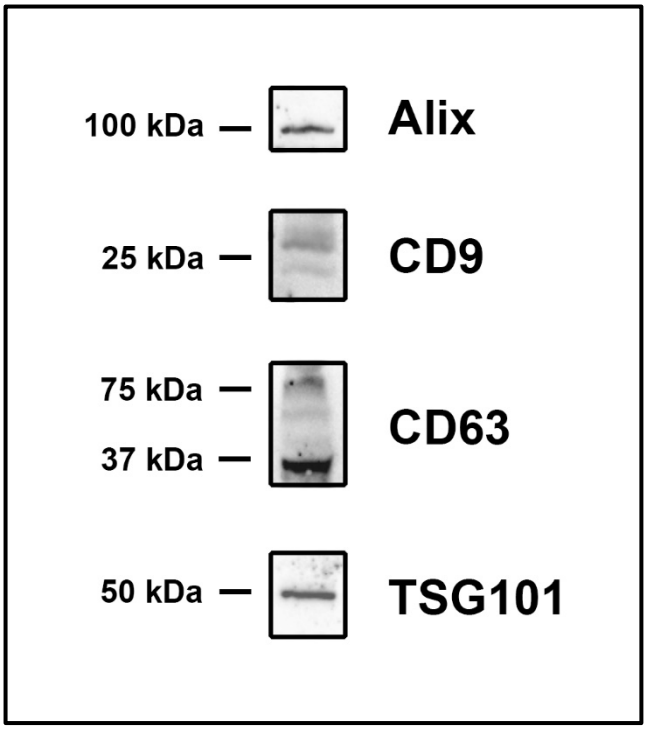


Figure S5*. Western Blot analysis of EVs isolated from HEK-293 cell culture media by ultracentrifugation.*

2.3 TEM Microscopy: Transmission electron microscopy (TEM) was performed on isolated EVs, resuspended in PBS, to analyze their ultrastructural morphology. According to proper dilution, the sample was adsorbed to 300 mesh carbon-coated copper grids (Electron Microscopy Sciences, Hatfield, PA, USA) for 5 min in a humidified chamber at room temperature. EVs on grids were then fixed in 2% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) in PBS for 10 min and then briefly rinsed in milli-Q water. Grids with adhered sEVs were examined with a Philips CM 100 transmission electron microscope TEM at 80kV, after negative staining with 2% phosphotungstic acid, brought to pH 7.0 with NaOH. The images were captured by a Kodak digital camera.

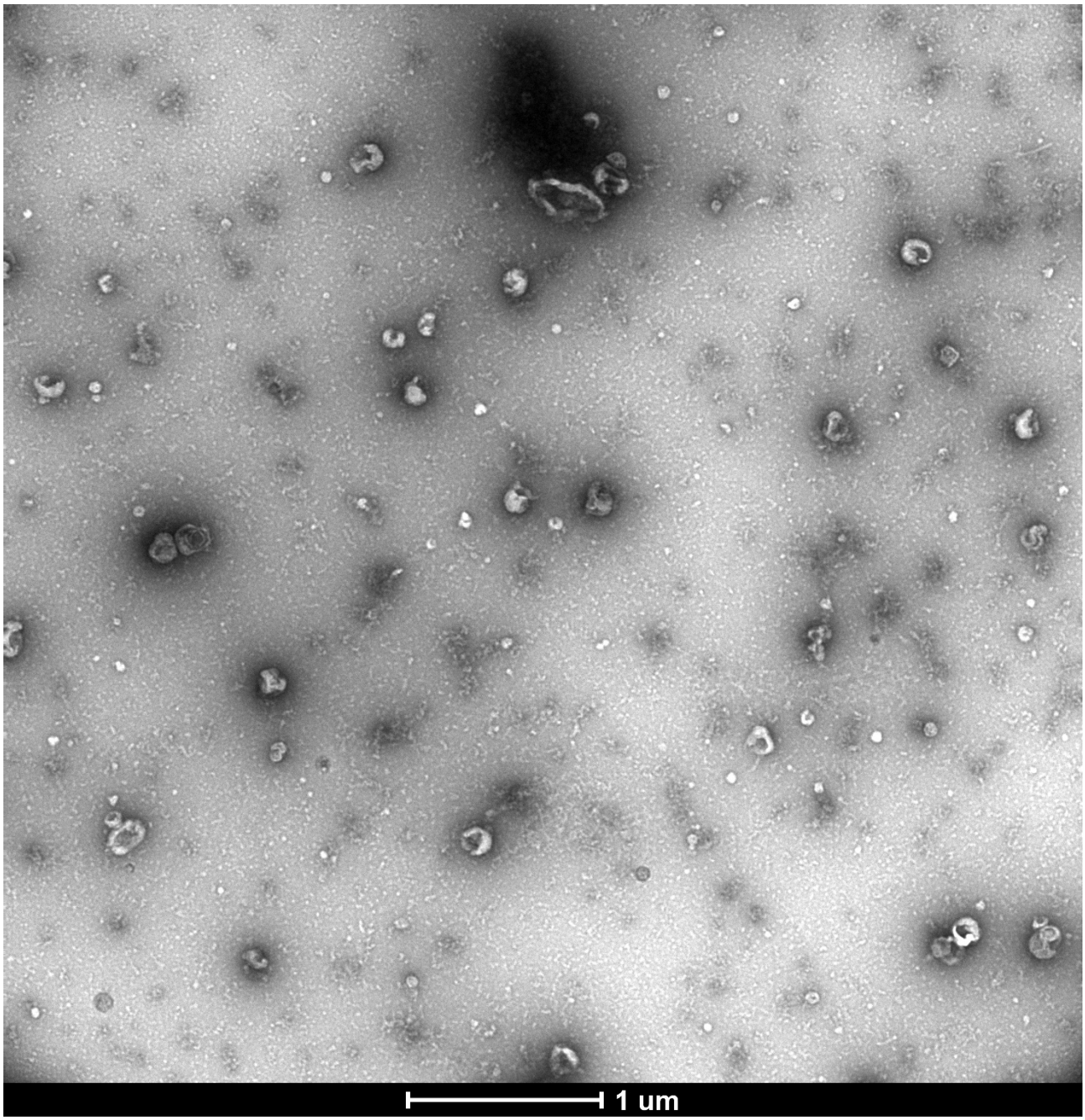


Figure S6*.TEM microscopy image of EVs isolated from HEK-293 cell culture media by ultracentrifugation. Scale bar indicates 1 µm.*

References

[1] D. Brambilla, A. Mussida, A.M. Ferretti, L. Sola, F. Damin, M. Chiari, Polymeric Coating of Silica Microspheres for Biological Applications: Suppression of Non-Specific Binding and Functionalization with Biomolecules, Polymers (Basel). 14 (2022). https://doi.org/10.3390/POLYM14040730.

[2] E. Chiodi, F. Damin, L. Sola, L. Ferraro, D. Brambilla, M.S. Ünlü, M. Chiari, A Reliable, Label Free Quality Control Method for the Production of DNA Microarrays with Clinical Applications, Polym. 2021, Vol. 13, Page 340. 13 (2021) 340. https://doi.org/10.3390/POLYM13030340.