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Competitive binding assay for biotin and biotin derivatives, based on avidin and biotin-4-fluorescein

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Abstract

Biotinylated molecules are extensively employed in bioanalytics and biotechnology. The currently available assays for quantification of biotin groups suffer from low sensitivity, low accuracy, or provide highly variable responses for different biotin derivatives. We developed a competitive binding assay in which avidin was pre-blocked to different extents by the biotinylated analyte and a constant amount of biotin-4-fluorescein (B4F) was added, resulting in strong quenching of the B4F. The assay was robust and the shape of the titration curve immediately revealed whether the data were reliable or perturbed by steric hindrance in case of large biotin derivatives. These advantages justified well the 10× higher sample consumption (~0.6 nmol) compared to single point assays. The assay was applied to a representative set of small biotin derivatives and validated by cross-control with the well-established 2-anilinonaphthalene-6-sulfonic acid (2,6-ANS) binding assay. In comparison to the 2,6-ANS binding assay, the lower precision (±10%) was compensated by the 100-fold higher sensitivity and the deviations from the ANS-assay were ≤5%. In comparison to the more sensitive biotin group assays, the new assay has the advantage of minimal bias for different biotin derivatives. In case of biotinylated DNA with 30 nucleotides, steric hindrance was found to reduce the accuracy of biotin group determination; this problem was overcome by partial digestion to n≤5 nucleotide residues with a 3'-exonuclease. The newly proposed biotin group assay offers a useful compromise in terms of sensitivity, precision, trueness, and robustness.

Keywords:

Avidin, biotin assay; biotin-4-fluorescein; biocytin; fluorescence; titration.

1. Introduction

Biotin is the prosthetic group of many carboxyl transferases in prokaryotes and eukaryotes. At the same time, biotinylated molecules are popular tools in bioanalytics and medical diagnostics (Dundas, Demonte, & Park, 2013), due to their high affinity and almost irreversible binding towards (strept)avidin (Green, 1990). For these reasons a number of assays have been developed by which biotin, its metabolites, and biotinylated molecules can be quantified over a wide range of concentrations, as reviewed in ref. (Livaniou et al., 2000) and further exemplified by recent studies (Ikeda, Miyao, & Sueda, 2014; Indyk, Gill, & Woollard, 2014; Rispens & Ooijevaar-de Heer, 2016).

Bioassays (Baur, Suormala, Bernoulli, & Baumgartner, 1998) and solid phase assays with radiolabels (Baur et al., 1998; Groman, Rothenberg, Bayer, & Wilchek, 1990; D. M. Mock, 1990) or marker enzymes (Bayer & Wilchek, 1990; D. M. Mock, 1997; Staggs, Sealey, McCabe, Teague, & Mock, 2004) provide for very high sensitivity but they are time-consuming and their precision is moderate. In comparison, homogenous biotin assays are less sensitive but more convenient and usually more precise. In the classical example (Green, 1965), avidin is mixed with an excess of 4'-hydroxyazobenzene-2-carboxylic acid (HABA, K_d = 5.8 µM) which occupies most biotin-binding sites whereupon it shows high absorbance at 500 nm. Step-wise titration of this mixture with biotin results in a linear decrease of A_{500}, up to the point where all binding sites are occupied with biotin and A_{500} remains constant upon further addition of biotin (Green, 1965). Due to its linear dose response, high accuracy, and simplicity, the HABA assay is still used in most commercial kits for biotin determination, whereby the biotin consumption has been reduced from ~40 to ~4 nmol biotin groups by switching from cuvettes to microtitre plates (MTP) and by measuring only the initial linear slope (e.g. in the biotin quantitation kits from Abcam or from Pierce (Thermo Fischer Scientific)). Alternatively, HABA has been replaced by the fluorescent probe 2-

anilinonaphthalene-6-sulfonic acid (ANS), providing for a fluorescence assay which closely resembles the HABA assay with respect to its linear dose-response, its high accuracy, and its moderate sensitivity (D. M. Mock & Horowitz, 1990).

A linear signal decrease is also observed if the endogenous tryptophan fluorescence of (strept)avidin is used to monitor binding of biotin (Der-Balian, Gomez, Masino, & Parce, 1990; Lin & Kirsch, 1977, 1979). The sensitivity is ~100× higher than in the HABA and ANS assays but (because of fluorescence excitation at 290 nm) this assay cannot be used when significant amounts of other proteins are present. Moreover, it is not known whether it works equally well with various derivatives of biotin.

The most sensitive fluorescence assay for biotin is based on the discovery that avidin is preferentially labeled by fluorescein-isothiocyanate (FITC) next to the four biotin-binding sites and that the bound FITC residues are significantly quenched in this location (Al-Hakiem, Landon, Smith, & Nargessi, 1981). Binding of biotin abolishes the quenching and results in >100% fluorescence increase (Al-Hakiem et al., 1981; Nargessi & Smith, 1986). FITC-labeled streptavidin is even more responsive, showing >160% fluorescence enhancement upon binding of biotin (Barbarakis, Smith-Palmer, Bachas, Chen, & Van Der Meer, 1993). The most dramatic effect is seen with Alexa488-labeled avidin if the Alexa488 labels are additionally quenched by bound HABA; thereby the addition of biotin causes displacement of HABA and results in an almost 20-fold fluorescence increase (Batchelor, Sarkez, Cox, & Johnson, 2007). These assays are simple, precise and can determine few picomoles of biotin. Unfortunately, though, even closely similar biotin derivatives (such as biocytin) give very different responses from biotin. Therefore, the assay must always be calibrated with the biotin derivative of interest (Barbarakis et al., 1993; Batchelor et al., 2007) and mixtures of different biotin derivatives can only be analyzed by a combination of reversed-phase chromatography and post-column detection with FITC-streptavidin (Przyjazny, Hentz, & Bachas, 1993; Rao,

Anderson, & Bachas, 1997). Another drawback of FITC-avidin-based assays is their non-linear dose response and lack of a defined breakpoint associated to the state where exactly four biotin groups are added per (strept)avidin tetramer. This behavior is obviously due to the imperfect site-specificity of (strept)avidin labeling with FITC. No such problems were seen in case of site-specific incorporation of non-natural amino acids like methoxycoumarine-alanine or –glutamic acid in position 120 of streptavidin (Murakami, Hohsaka, Ashizuka, Hashimoto, & Sisido, 2000) but unfortunately the engineered streptavidins with the methoxycoumarine labels are not commercially available.

The goal of the present study was to develop a homogeneous fluorescence assay for biotin derivatives which meets the following criteria: (i) The reagents for the assay should be commercially available. (ii) The sample consumption should not be higher than in the tryptophan quenching assay (~0.5 nmol). (iii) In contrast to the latter, the wavelengths of excitation and emission should be in the visible region to minimize background fluorescence. (iv) The dose-response should be linear, with a clear-cut breakpoint when all biotin-binding sites in avidin are saturated with biotinylated analyte molecules.

Being in need of such an assay, we remembered a published feasibility test which had met criteria (i)-(iv) (Ebner et al., 2008). The assay principle takes advantage of the irreversible binding of biotin (or a biotin derivative) to avidin, and of the fact that subsequently added B4F occupies the remaining sites and gets quenched, while the excess of unbound B4F molecules shows undiminished fluorescence (see Fig. 1).

In the present study we screened for the optimal assay parameters and checked whether the same linear dose-response is also found with various kinds of biotin derivatives. We also addressed steric problems of larger biotinylated molecules that might prevent saturation of all four binding sites per avidin tetramer under the given assay conditions. The new assay was

validated by comparison with the ANS binding assay, taking advantage of its high accuracy at higher analyte concentrations.

2. Materials and methods

UV/vis measurements were conducted on a Hitachi U-3010 spectrophotometer, all fluorescence measurements were performed on a Hitachi F-2700 fluorimeter. Details on used materials and procedures can be found in the Supplements.

2.1. Buffers

Buffer A (100 mM NaCl, 50 mM Na_2HPO_4, 1 mM EDTA, pH adjusted to 7.5 with NaOH) and buffer B (1 mg ml^{-1} BSA in buffer A) were used in all titration experiments, as specified.

2.2. Stock solutions of fluorescent probes

ANS was dissolved in DMSO to give a 5 mM stock solution. HABA was dissolved in alkaline aqueous solution to give a 20 mM stock solution. B4F was dissolved in DMSO to obtain a concentration of approx. 500 µM ("nominal concentration by weight", NCBW). Further dilution with buffer A yielded an approx. 20 µM stock solution (NCBW). The effective concentration (EC) of the B4F stock solution was determined to be 16.0 µM (Fig. S2) (Kada, Falk, & Gruber, 1999). The nominal concentration of B4F deviates from the effective concentration because of imperfect purity of commercial B4F (Kada, Falk, et al., 1999). B4F was diluted to 0.160 µM (EC) with buffer B immediately before its use in a B4F binding assay.

2.3. Stock solutions of biotin and biotinylated compounds

Except for biotinylated DNA, all concentration values stated in this section are "nominal concentrations by weight" (NCBW), with the provisional assumption that the compound had a

purity of 100%. All stock solutions were diluted with buffer A to 400 µM for the ANS assay and with buffer B to 0.2 µM for the competitive B4F assay, respectively.

D-biotin was dissolved in an aqueous solution of 100 mM Tris base to yield a 4 mM stock.

Biocytin was dissolved in an acetic acid-water mixture and, if necessary, diluted with buffer A to a final stock concentration of 1 mM or 4 mM.

Biotin-PEG_8-amine was converted into the trifluoroacetic acid (TFA) salt and taken to dryness, while biotin-C_3-amine was already available as TFA salt. Both compounds were dissolved in DMSO at a concentration of 4 mM.

The TFA salts of biotin-PEG_1-amine and biotin-PEG_2-amine were both purchased as DMSO stock solutions with a declared concentration of 25 mg ml^{-1}. For the competitive B4F binding assay, aliquots of the DMSO stock solution were first diluted with 100 mM NaH_2PO_4 to a concentration of 50 µM or 100 µM, respectively, ensuring protonation by this acidic buffer.

5'-Biotin-DNA was dissolved in water to give a 100 µM solution. Further dilution with buffer A yielded a 4 µM stock solution. Determining the absorption A_{260} of a 10-fold diluted sample gave a concentration value of 0.413 µM in the diluted sample (ε_{260}= 306×10^3 M^{-1} cm^{-1}, OligoCalc (Kibbe, 2007)), thus a concentration of 4.13 µM in the stock solution.

A sample of 5'-biotin-DNA was subjected to treatment with exonuclease I in order to receive a digested sample (see section S4.)

2.4. Stock solutions of avidin

For the HABA and ANS assays, avidin concentration was 3 µM tetramers (NCBW, batch 1). For determination of the optimal assay conditions (section 2.7), avidin "working solution No.

1" (0.105 µM (EC)) was used. For the competitive binding assays, avidin "working solutions No. 2" (final concentration: 34.8 nM (EC) in batch 2 and 33.4 nM (EC) in batch 3) were used.

2.7. Titration of B4F with avidin to determine the optimal competitive B4F assay conditions

Triplicates of 12 polystyrene tubes (5 ml) were prepared containing increasing amounts of avidin (working solution No. 1) and a constant amount of B4F (0.160 µM) in a constant final volume of 1 ml. For this purpose, 1000 - *x* - 100 µl buffer B was mixed with *x* µl of avidin working solution No. 1 (*x* = 0, 10, 20, 30, 40, 50, 60, 80, 100, 120, 140, 160 µl). Then, 100 µl of 0.160 µM B4F was added to each tube and each tube was vortexed immediately after the addition. After incubation for 10 min in the dark, the fluorescence at 525 nm (5 nm slit) was measured while exciting at 490 nm (2.5 nm or 5 nm slit). The fluorescence values were plotted against the added volume of avidin working solution No.1. A linear fit was applied to the segment which showed a steep initial drop, as well as to the moderate subsequent increase of the titration curve (see Fig. 4 for batch 2 and Fig. S7 for batch 3). The intersection point of the two lines gave the volume of the avidin working solution (in µl) at which all B4F was bound to avidin.

The purpose of the above described B4F titration with avidin was to find out how much avidin was necessary to bind 90% of the B4F molecules in each assay tube; this was the desired working condition for the competitive B4F binding assay.

In batch 2 (Fig. 4) the breakpoint occurred at 37.01 µl of 0.105 µM avidin tetramer (3.87 pmol). From this result, the desired concentration of the avidin "working solution No. 2" was calculated as 0.9 × 3.87 pmol / 100 µl = 34.8 nM. In batch 3 (Fig. S7), the breakpoint was at 35.6 µl of 0.105 µM avidin tetramer (3.72 pmol), thus concentration of "working solution No. 2" was calculated as 0.9 × 3.72 pmol/100 µl = 33.4 nM avidin tetramer.

2.8. Competitive binding assay with biotin-4-fluorescein

Typically, triplicates of 17 polystyrene tubes (5 ml) were prepared containing increasing amounts of *D*-biotin (or a biotin derivative) and constant amounts of avidin and B4F. First, 1000 - *x* - 200 µl buffer B was mixed with *x* µl of 0.2 µM biotin-reagent (*x* = 0, 5, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130 µl). Then, 100 µl of "avidin working solution No. 2" was added and each tube was vortexed immediately after the addition of avidin. After incubation for 10 min, 100 µl of 0.160 µM B4F was added to each tube. After incubation for 10 minutes in the dark, the fluorescence of the samples was measured at 525 nm (5 nm slit) while irradiating at 490 nm (5 nm slit).

The fluorescence values were plotted against the volume (in µl) of the added biotin-reagent solution (Figs. 5, 6 and Fig. S8 with avidin batch 2, Fig. S9 with avidin batch 3). A linear fit was applied to the two linear segments, the steep initial rise and the plateau at high biotin concentration using the piecewise fitting function “PWL2” of the software Origin® (OriginLab, Northhampton). The obtained intersection point (x_i) indicated the volume at which all binding sites were saturated by biotin residues. When multiple titration curves were measured, the mean value of the intersection point was calculated and the error was computed as the standard deviation of the obtained x_i values.

3. Results and discussion

3.1. Use of the ANS assay for accurate characterization of the biotinylated test compounds

The goal of the present study was to establish the B4F-based biotin assay illustrated in Fig. 1 and to test it with a variety of biotin derivatives. Besides several small molecules, a longer polyethylene glycol (PEG) derivative and a 30-nucleotide biotinylated single-stranded DNA molecule were chosen. Due to the imperfect purity of most test samples, it was first necessary to determine their true biotin content.

The most accurate methods for biotin group determination are the HABA assay (Green, 1965) and the ANS assay (D. M. Mock & Horowitz, 1990; D. M. Mock, Lankford, & Horowitz, 1988). Testing their performance (Fig. 2), breakpoints occurred at the same biocytin consumption in the HABA and ANS assay (50.4 µl and 50.2 µl, respectively), proving full equivalence of the two assays.

Except for biotin-5'-DNA due to lack of material, the biotin content of all biotin derivatives was then tested by the ANS assay (Fig. 3). As expected for samples with different purity, the breakpoint did not occur at the same consumption of biotin derivative solution (see Table 1 and Table S1). The lowest consumption was observed with nominally 400 µM *D*-biotin (49.6 ± 0.6 µl, second column in Table 1 and Table S1), indicating that *D*-biotin was of highest purity among the tested samples. For this reason, *D*-biotin was selected as standard in the ANS assay.

The biotin content of the different compounds was calculated by dividing the breakpoint for the biotin standard (46.9 µl) by the breakpoint of the particular biotin derivative (third column in Table S1). Biocytin is a preferred standard when determining the biotin group content of biotinylated proteins (Oberoi, 2014; Przyjazny et al., 1993; Rao et al., 1997). *D*-biotin is less convenient to use as standard because it can sometimes degrade on a time scale of hours at

room temperature (Ebner et al., 2008) and it is completely destroyed during the HCl digestion of proteins (unpublished results). For these reasons we decided to henceforth use biocytin as standard, taking into account its biotin content of 97.7%.

3.2. Concept of the new competitive B4F binding assay

The main drawback of quantifying the biotin content by ANS displacement is the high consumption of sample. One measurement requires ~100 µl of approximately 400 µM biotinylated compound (i.e., 40 nmol). We therefore set out to establish a more sensitive homogeneous assay.

The principles of the new biotin assay are illustrated in Fig. 1. In a series of tubes (or wells), avidin is always mixed with exactly the same amount of biotin-4-fluorescein (B4F). The amount of B4F exceeds the amount of binding sites by ~10%. This means that in absence of a biotinylated analyte (panel A) ~90% of B4F molecules are bound and strongly quenched (Kada, Falk, et al., 1999). In presence of a small amount of biotinylated analyte (panel B), only the remaining binding sites in avidin can be occupied by B4F, leading to a higher fraction of free and fluorescent B4F. Panel C illustrates the situation when the sample contains just the right amount of biotinylated analyte to saturate all binding sites in avidin. Here, the addition of B4F leads to maximal fluorescence because all B4F molecules are unbound and unquenched. No further fluorescence increase is seen when mixing avidin with higher amounts of biotinylated analyte before adding B4F (panel D).

A feasibility test of this competitive B4F binding assay had been reported with *D*-biotin as the analyte (Ebner et al., 2008). In the present study the assay conditions and the data evaluation method were standardized and the general applicability to different biotin derivatives was tested as described below.

3.3. Adjustment of the working conditions for the competitive B4F binding assay

The reasons for choosing a 10% excess of free B4F over the number of avidin's ligand-binding sites in the assay mixture are the following: (i) It is essential to avoid any excess of binding sites over B4F because otherwise small amounts of a biotinylated analyte would not cause a response of the assay. (ii) It would be difficult to reproducibly adjust an exact match between binding sites and B4F in each assay tube. (iii) The 10% fraction of unbound B4F in the blank sample is important to ensure fast association of B4F with avidin within the standard incubation time (10 min).

The method for finding the proper assay condition is shown in Fig. 4. In parallel tubes, a constant amount of B4F (16 pmol) was mixed with increasing amounts of avidin in a final volume of 1 ml. As expected (Kada, Kaiser, Falk, & Gruber, 1999), this titration resulted in a linear decrease of B4F fluorescence, with a sharp minimum at 37.0 µl when the number of binding sites matched the number of B4F molecules in the sample.

It is of utmost importance to use the same amount of B4F (16 pmol per tube) in the pre-test (Fig. 4) as in the final assay (Figs. 5-6), as well as a constant amount of avidin which binds 90% of B4F. According to Fig. 4, the proper volume of the "avidin working solution No. 1" was $0.9 \times 37.0 = 33.3$ µl. Using 100 µl volume for convenience and accuracy, the "working solution No. 1" was diluted 100/33.3, resulting in "working solution No. 2".

Of note, the "effective concentration" values of the avidin (34.8 nM) and B4F (160 nM) solutions are only used for adjustment of the proper working conditions. These numbers have no influence on the evaluation of the final B4F binding assay because the latter is calibrated with a certified standard.

3.4. Determination of biotin groups by the competitive B4F binding assay

The calibration of the B4F binding assay is presented in Fig. 5. In each titration experiment, a constant amount of avidin was mixed with increasing amounts of biocytin, followed by the addition of a constant amount of B4F which represented a 10% excess over the number of binding sites. In absence of biocytin the fluorescence was minimal, as expected for maximal binding of B4F. Increasing amounts of biocytin caused a linear rise in B4F fluorescence up to complete saturation of avidin with biocytin, followed by a constant high fluorescence level when the amount of biocytin exceeded the number of binding sites in the sample. The linear rise and the horizontal plateau were fitted by straight lines and the intersection point was determined using the piecewise fitting procedure of Origin®.

The biocytin solution used in Fig. 5 had a nominal concentration of 0.200 µM, corresponding to an effective concentration of 0.195 µM according to the ANS assay data (Table 2). Consequently, the average breakpoint for six titration experiments at 43.4 µl ± 3.1 µl of 0.195 µM biocytin standard indicated that with this batch of avidin working solution No. 2 the breakpoint always occurred when 8.49 pmol of a biotin derivative are present in the sample (Table S2).

Subsequently, the same titration as in Fig. 5 was performed with nominally 0.200 µM solutions of biotin-PEG_1-amine and biotin-PEG_8-amine (Fig. S8). Division of 8.49 pmol by the volume of biotin derivative consumed at the intersection point gave the effective biotin concentration in the nominally 0.200 µM biotin derivative solution (last column in Table 2, batch 2).

The analogous procedure was performed with a separate batch of the "avidin working solution No. 2". Here, the calibration gave a breakpoint at 40.8 ± 2.3 µl 0.195 µM biocytin (7.97 pmol, Fig. S9A, Table S2, batch 3) and this batch was used to determine the effective biotin content

in nominally 0.200 µM solutions of biotin-C_3-amine and biotin-PEG_2-amine (Fig. S9, Table S2).

As illustrated in Fig. 1, the B4F binding assay relies on blocking of all four binding sites in avidin by the biotinylated analyte. In this respect, problems were expected with large analytes, such as DNA. We thus tested single-stranded biotin-5'-DNA composed of 30 nucleotides (9199 g/mol, Fig. 6A). When avidin was titrated with this biotin-5'-DNA, a quasi-linear rise and a breakpoint were observed but the usual plateau after the breakpoint was no longer horizontal. We took this as an indication of mutual steric hindrance when four large biotin-5'-DNA molecules were to be bound per avidin molecule.

Digestion of an aliquot of the same biotin-5'-DNA with a 3'-exonuclease resulted in single strands with ≤5 nucleotides. The proper digestion conditions were found by digesting the analogous TMR-5'-DNA and analyzing the resulting fragment size by gel filtration (Figs. S3 and S4). After the digestion of biotin-5'-DNA, the B4F binding assay showed a much more horizontal plateau (Fig. 6B) but the consumption of biotin-5'-DNA was still very similar as before the digestion (Fig. 6A), as also reflected in the derived "concentration by B4F assay" (CBB) for undigested and digested biotin-5'-DNA (last column in Table 2). As expected, these numbers show that digestion of biotin-DNA gives a narrower random error in the B4F binding assay, although the extent of deviation in the breakpoint with undigested biotin-DNA was not as large as expected.

The locations of the breakpoints in Fig. 6A and 6B indicated binding of four, rather than only two, biotin-5'-DNA molecules per avidin tetramer. This can be concluded from the comparison between the nominal concentration of the biotin-5'-DNA solution (0.200 µM according to A_{260}, third column in Table 2) with the concentration measured by the B4F binding assay (0.235 µM and 0.227 µM, last column). The deviation from the concentration

calculated from A_{260} is attributed to the fact that the molar absorptivity (ε_{260} = 306×10^3 M^{-1} cm^{-1}) obtained by OligoCalc is an estimate (Kibbe, 2007).

A comparison between the well-established ANS assay and the more sensitive B4F assay is presented in Table 1. Biocytin with a biotin content of 97.7% was used as the common standard, therefore its recovery in the B4F assay (i.e., the agreement with the ANS assay) was defined as 100% (last column in Table 1). For the other biotinylated analytes, the relative deviation of the B4F assay from the ANS assay was within the range of ±5%.

The major advantage of the new B4F-based titration assay was much lower sample consumption. One HABA or ANS titration requires 100 µl of 400 µM biotinylated analyte (40 nmol), whereas one complete B4F titration required <0.6 nmol of sample (17×3 tubes with an average dosage of <80 µl 0.2 µM biotin per tube, see Fig. 5). A further 10-fold reduction in sample consumption in the new B4F assay could be achieved by switching from cuvettes to the MTP format as realized in commercially available HABA assay kits. Alternatively, the sample consumption could further be reduced by working at lower avidin concentrations in all three above mentioned assays. This strategy, however, not only implies a lower signal-to-noise ratio but also a loss of the clear-cut breakpoint in case of larger biotinylated analytes (Kaiser, Marek, Haselgrübler, Schindler, & Gruber, 1997).

The tradeoff for higher sensitivity of the new B4F assay, as compared to the ANS assay, is the lower precision of the B4F assay (see Table 1). This is in part attributed to the multiple pipetting steps in the latter method.

Most importantly, the B4F assay data were in good agreement with the reliable results from the ANS assay, expressed as "recovery" in the last column of Table 1. The "trueness" of the B4F assay originates from the assay principle: As in the HABA and ANS titration assays, the amount of biotinylated analyte is not directly calculated from a measured signal but from the

consumption required for saturation of all binding sites in avidin, which is the breakpoint in the fluorescence profile. This makes the B4F assay robust to very high background color and background fluorescence of the biotin sample, as already shown in the first feasibility test of this assay (Ebner et al., 2008).

4. Conclusions

The goal of this study was to establish a biotin group assay which requires much less sample than the HABA and ANS assay, while retaining the high robustness and reliability of these conventional biotin group assays. This goal has been successfully reached with the new titration assay where a standardized avidin solution is progressively saturated with the biotinylated analyte, and a constant amount of B4F is added to determine the fraction of unoccupied binding sites. The sample consumption is two orders of magnitude smaller than in the HABA or ANS titration assays but one order of magnitude higher than when using FITC-labeled (strept)avidin as the probe molecule. The new B4F binding assay shows a linear dose-response and a clear-cut breakpoint when all binding sites are saturated with analyte. Consequently, the new assay can be calibrated with biotin (or biocytin) and does not require calibration with the biotin derivative of interest. In conclusion, the new B4F assay provides for a balanced compromise in terms of sensitivity, precision, trueness, and easy calibration. Furthermore, the new assay is insensitive for the background fluorescence signal, making it attractive choice for colorful samples.

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Tables

Table 1. Determination of the biotin group contents of biotin derivatives by the ANS and B4F assay.

biotin derivative	breakpoint [a] (µl)	biotin content ANS assay [b] (%)	biotin content B4F assay [c] (%)	recovery in B4F assay [d] (%)
D-biotin	49.6 ± 0.6	≡100.0 ± 1.2 [e]	n.d.	n.d.
biocytin	50.7 ± 0.9	97.7 ± 1.8	≡98 ± 7 [f]	≡100 ± 7 [f]
biotin-C_3-amine	56.9	87.1	86 ± 3	99 ± 3
biotin-PEG_1-amine	50.8	97.6	93 ± 3	95 ± 3
biotin-PEG_2-amine	55.6 [g]	89.1 [g]	94 ± 4	105 ± 5
biotin-PEG_8-amine	51.2 [g]	96.9 [g]	97 ± 3	100 ± 3

[a] Calculated from the data in Table S1.

[b] Calculated from the data in Table S1, referring to *D*-biotin as standard.

[c] Calculated from the B4F assay data (Table 2) as 100% × CBB / NCBW.

[d] The recovery in the B4F assay was calculated from the ratio CBB/CBA (see last two columns of Table 2).

[e] *D*-Biotin was used as standard in the ANS assay. Therefore, its "biotin content" was set to 100% by definition, as indicated by the triple bar (≡).

[f] Biocytin was used as standard for the B4F assay. Therefore, its biotin content was set to 97.7% (as determined in the ANS assay) and its recovery was 100% by definition, as indicated by the triple bar (≡).

[g] Average value from two titrations.

Table 2. Measurement of the biotin content of various biotin derivatives by the competitive B4F binding assay.

biotin derivative	NCBW [a] (µM)	conc. from A_{260} (µM)	CBA [b] (µM)	CBB [c] (µM)
biocytin	0.200	-	0.195± 0.004	≡0.195 ± 0.014 [d]
biotin-C_3-amine	0.200	-	0.174	0.173 ± 0.006
biotin-PEG_1-amine	0.200	-	0.195	0.186 ± 0.006
biotin-PEG_2-amine	0.200	-	0.178	0.187 ± 0.009
biotin-PEG_8-amine	0.200	-	0.194	0.195 ± 0.006
5'-biotin-DNA	n.d. [e]	0.200	n.d. [e]	0.235 ± 0.021
5'-biotin-DNA, digested	n.d. [e]	0.207	n.d. [e]	0.227 ± 0.007

[a] "Nominal concentration by weight".

[b] "Concentration by ANS assay", calculated by multiplication of NCBW with the biotin content according to the ANS assay (Table 1).

[c] "Concentration by the B4F assay", determined as described in Table S2.

[d] Biocytin with the known biotin content of 97.7% (according to the ANS assay) was used as standard in the B4F assay. Therefore, the concentration of nominally 0.200 µM biocytin in the B4F assay was 0.195 µM by definition, as indicated by the triple bar (≡).

[e] The amount of 5'-biotin-DNA was too little for preparing a known concentration by weight and for the ANS assay.

Figures and figure legends

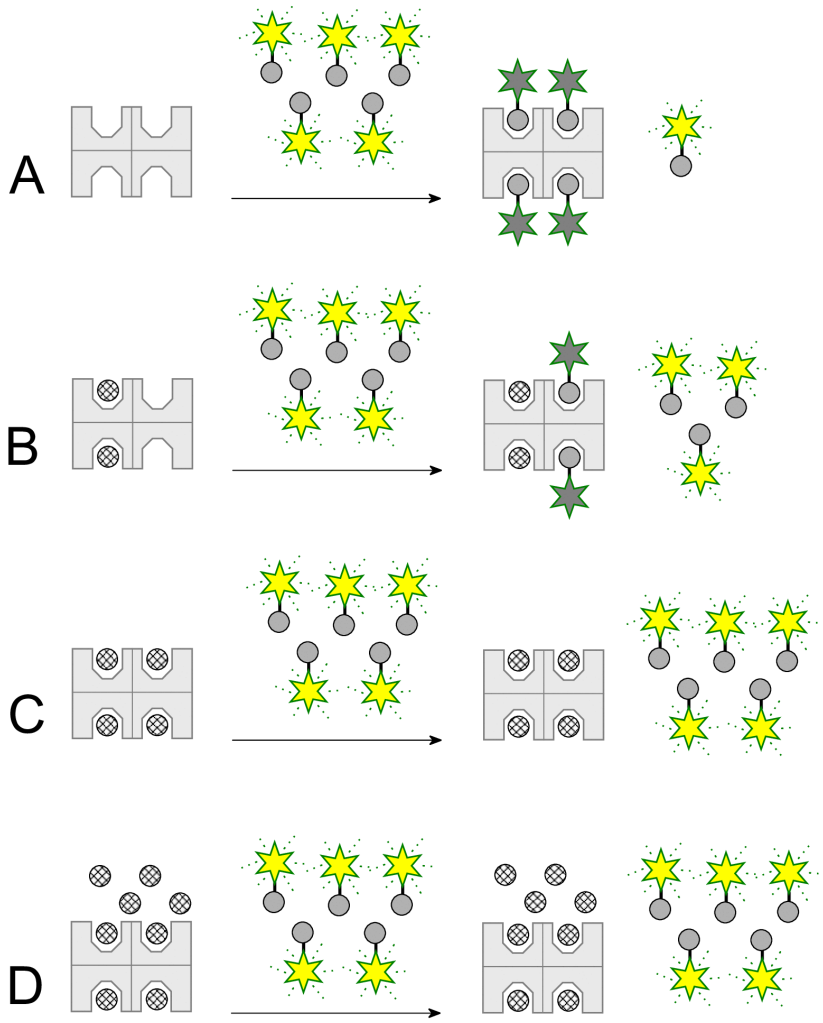

Fig. 1. Principle of the competitive B4F binding assay. Identical amounts of avidin are irreversibly blocked with increasing amounts of a biotin derivative and then always mixed with the same slight excess of B4F (~10%). (A) In absence of a biotin derivative, ~90% of the B4F is bound and quenched; only ~10% is free and fluoresces strongly. (B) Partial pre-blocking of avidin with a biotin derivative reduces both binding and quenching of B4F. (C) Saturation of avidin with equimolar biotin derivative prevents binding and quenching of all B4F molecules. (D) Due to irreversible and tight binding, an excess of biotin derivative has the same effect as the equimolar amount in (C).

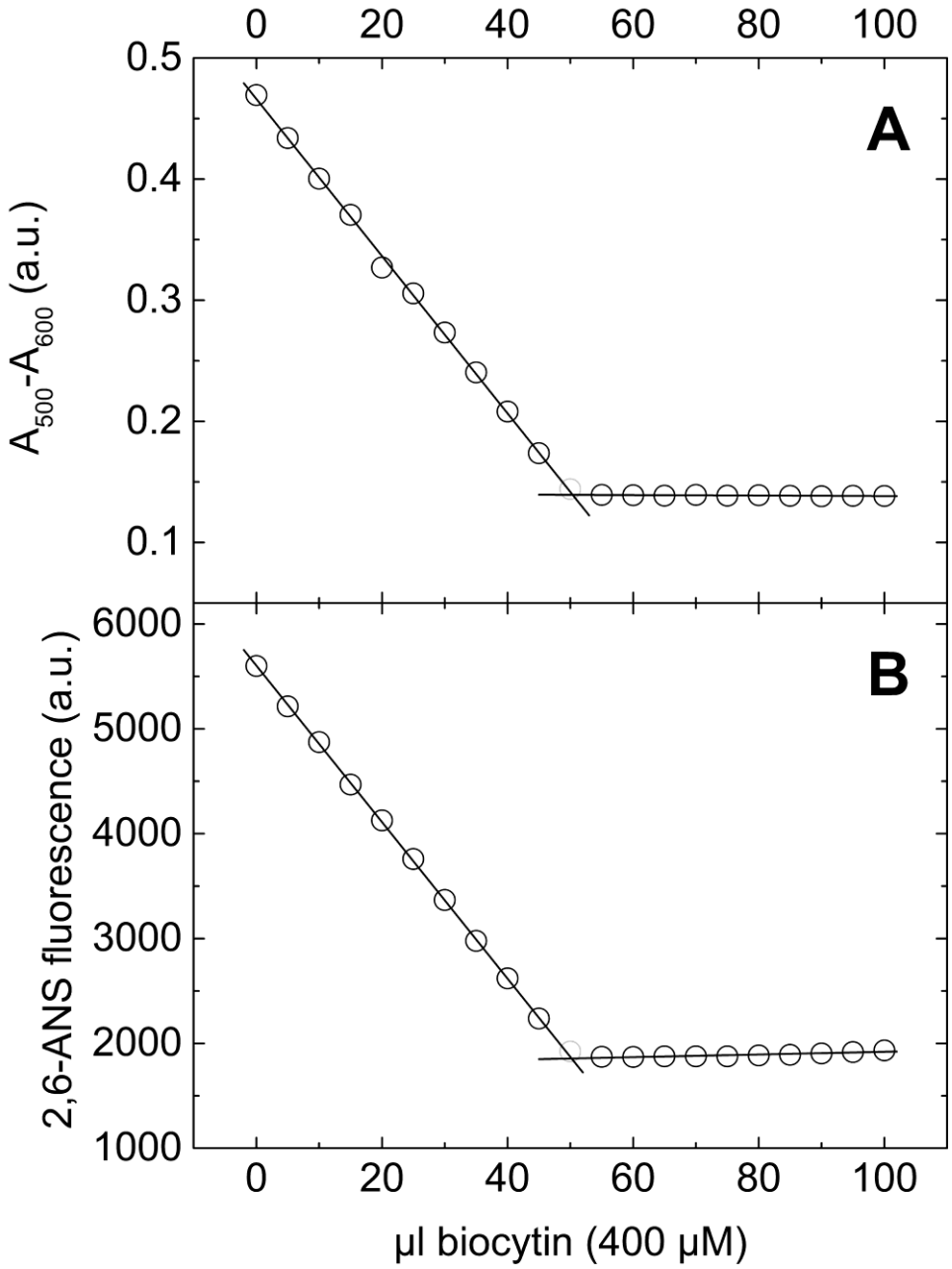

Fig. 2. Comparison of HABA (A) and ANS (B) as reporters for the titration of avidin with biocytin. 2 ml 3 µM avidin tetramer (NCBW) was mixed with HABA (20 µl, 20 mM) or ANS (25 µl, 5 mM) in a stirred cuvette and titrated with 400 µM biocytin (NCBW). The absorbance values (A_{500}-A_{600}) or the fluorescence signals (at 340 nm) were corrected for the dilution upon biocytin addition and plotted versus the consumption of biocytin. Data shown in grey was excluded from linear fitting. The intersection points (50.4 µl biocytin in (A) and 50.2 µl biocytin in (B)) reflected the amount of biocytin necessary for saturation of all binding sites in avidin sample.

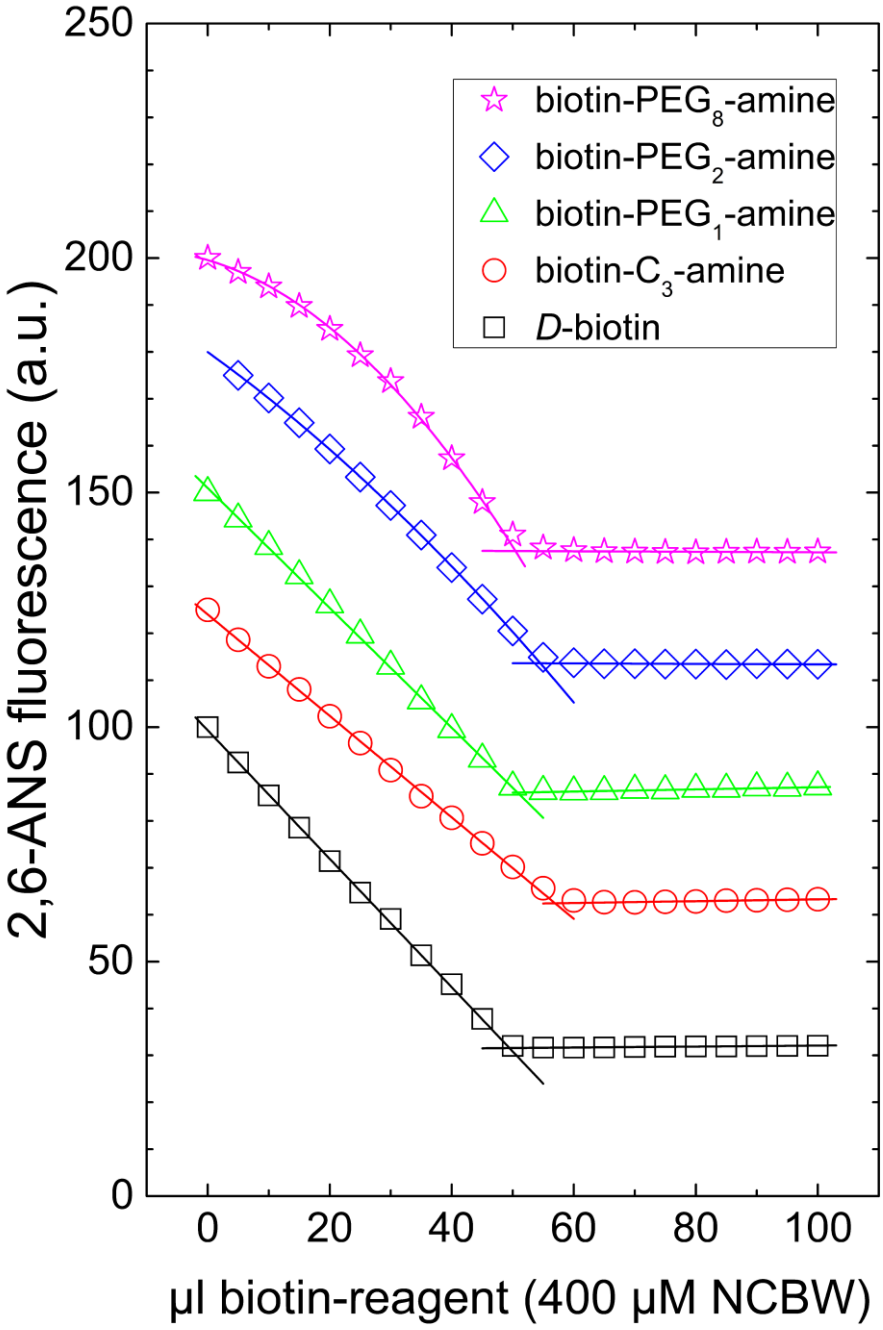

Fig. 3. ANS assay for *D*-biotin and biotin derivatives. As described in Fig. 1B, a 2 ml aliquot from the identical avidin solution (as in Fig. 2B) was mixed with ANS and titrated with 400 µM *D*-biotin or biotin derivative (NCBW). The data sets were processed as in Fig. 2B and the initial fluorescence of each curve was renormalized to 100 units. Except for *D*-biotin (□), all titration profiles were vertically displaced by multiples of 25 fluorescence units to avoid overlap of the data. The steep segments for biotin-PEG_8-amine and biotin-PEG_2-amine were analyzed by a polynomial fit ($y = A + B\,x + C\,x^2$), all other fits were strictly linear.

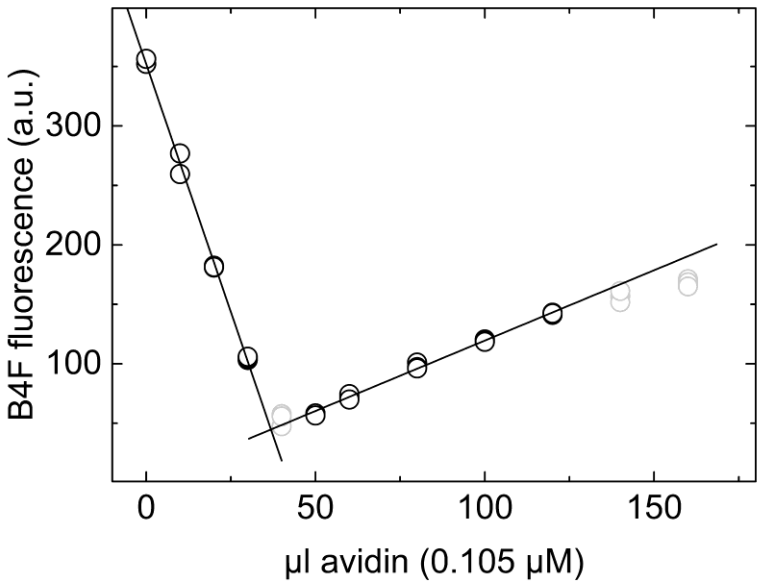

Fig. 4. Determination of optimal assay conditions by non-cumulative titration of a constant amount of B4F with increasing amounts of avidin. In a series of tubes, the specified volume (µl) of the 0.105 µM avidin working solution No. 1 (batch 2) was mixed with buffer B at a final volume of 900 µl and 100 µl 16.0 µM B4F was added. The resulting B4F fluorescence was plotted versus avidin consumption per tube. Data shown in grey was excluded from linear fitting. The intersection point (37.01 µl × 0.105 µM = 3.87 pmol avidin tetramer) indicated saturation of all binding sites by B4F. From this result, the desired concentration of the avidin "working solution No. 2" (batch 2) was calculated as 0.9 × 3.87 pmol / 100 µl = 34.8 nM.

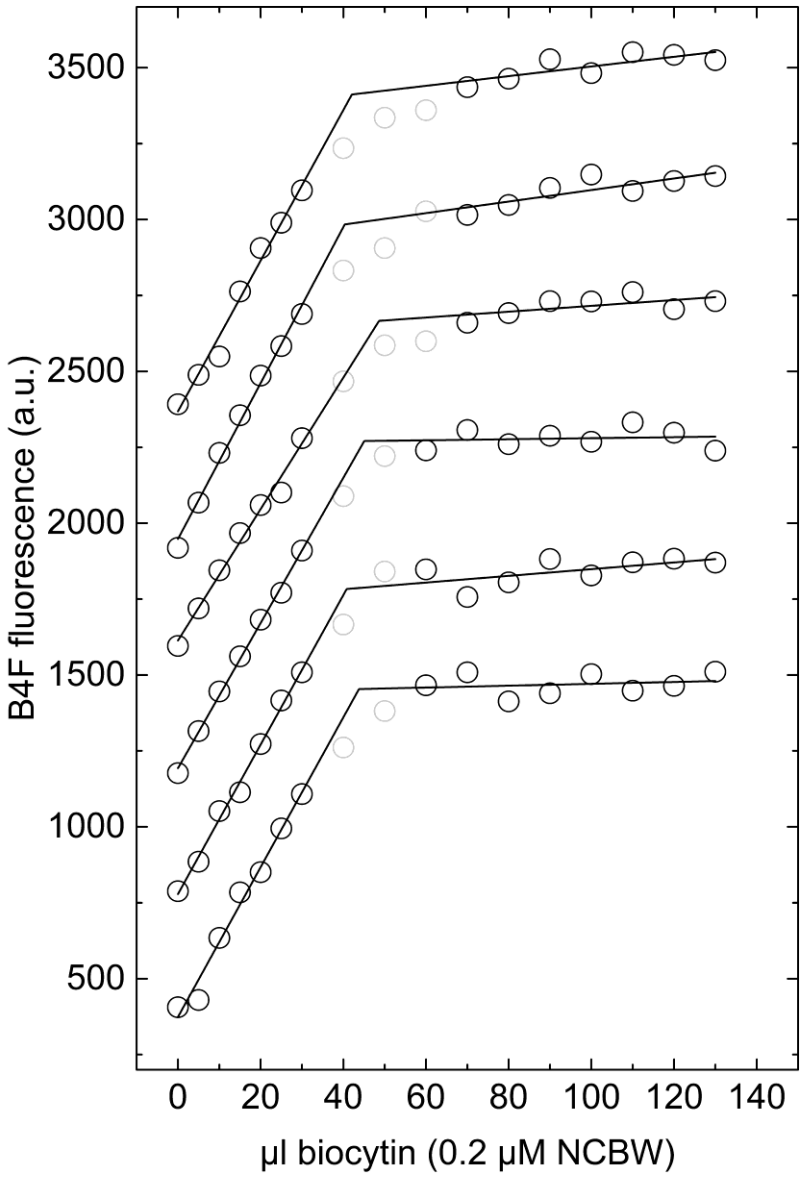

Fig. 5. Competitive B4F binding assay for determination of the biotin group content in the biocytin standard. In separate tubes, the specified volume of a nominally 0.2 µM biocytin solution was mixed with buffer B at a final volume of 800 µl. 100 µl of avidin working solution No. 2 (batch 2, 34.8 nM according to Fig. 4) was added. After 10 min, 100 µl 160 nM B4F was added and after another 10 min the fluorescence of B4F was measured. Data shown in grey was excluded from the linear fits. The intersection point (mean value 43.44 ± 3.10 µl) indicated saturation of all binding sites by avidin. In order to ensure better visibility, the individual titration curves were vertically stacked with an increment of 400 units.

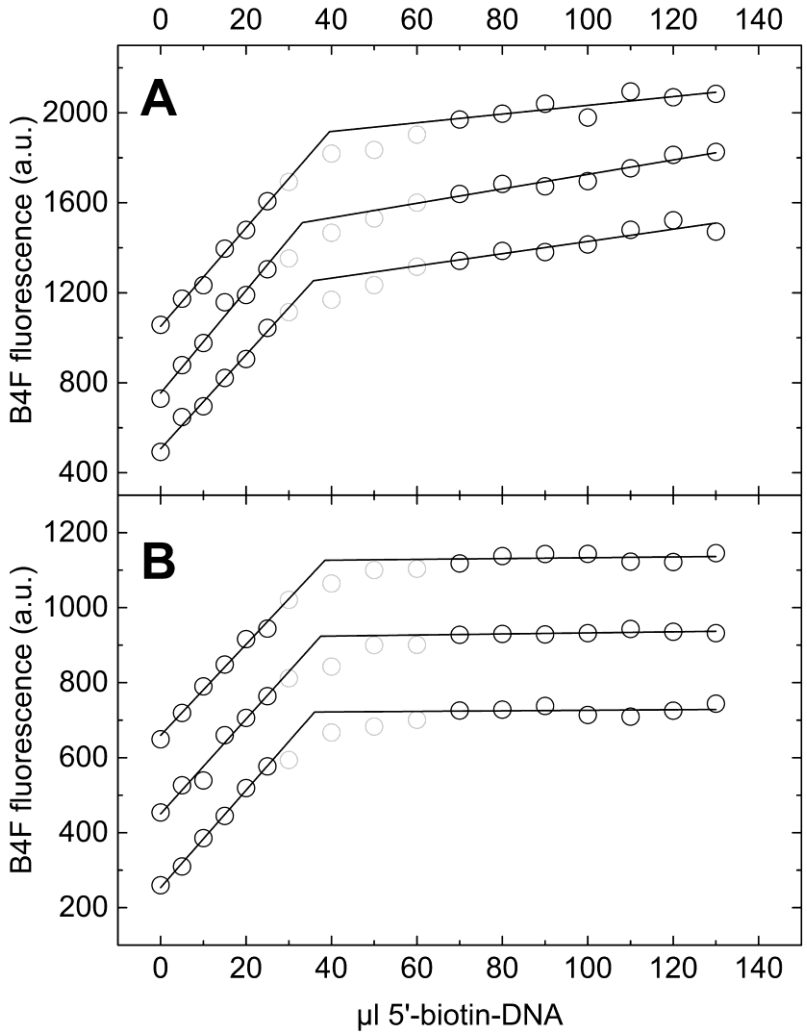

Fig. 6. Application of the competitive B4F binding assay to 5'-biotin-DNA (A) before digestion and (B) after digestion with exonuclease I. Undigested or digested 5'-biotin-DNA was mixed with buffer B at a final concentration of 0.200 µM or 0.207 µM, respectively (according to A_{260}). These working solutions were used for the analogous titration experiment as shown for biocytin in Fig. 5. All other details are the same as in Fig. 5. In order to ensure better visibility, the individual titration curves were vertically stacked with an increment of 300 units in A and 200 units in B, respectively.