MS63: The Potential of Eco-Genomic and Bioinformatic Approaches to the Study of Rotifers

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Abstract

Rotifers are a key component of many freshwater ecosystems, but large-scale temporal or geographical surveys of rotifer species and their communities are constrained by the labor-intensive process of species identification. The growing availability of molecular tools such as the polymerase chain reaction and DNA microarrays, and the ever-decreasing cost of DNA sequencing and of computer power, now provide a means of coupling traditional taxonomic identification with genomic and bioinformatic technologies. This combined approach could be used to track populations of specific rotifer species in space and time at a level of detail not possible with traditional methods, and is particularly well-suited to the study of interstitial or soil samples, including resting egg banks. An additional application would be rapid surveys of rotifers in new environments. While the DNA sequence information currently available would allow only a generalized identification of rotifers in such surveys, as the number of rotifer sequences accumulates, identification would become increasingly precise. Here we discuss two techniques, microarray hybridization and serial analysis of gene tags, that can be combined with traditional systematics to increase the breadth and sensitivity of ecological studies of rotifers and other micro-invertebrates. The power of these techniques would be maximized through collaborative efforts, which may be fostered through the data base of rotifer biology, WheelBase (http://jbpc.mbl.edu/wheelbase).

Introduction

Rotifers are found in large numbers in a wide variety of environments and play an important role in aquatic food webs (Hutchinson 1967; Williamson 1983; Arndt 1993; Rublee 1998; Ricci & Balsamo 2000; Wallace & Ricci 2002; Yakovenko, this volume). However, quantitative examination of rotifer abundance and species diversity is hampered by the difficulty of collecting samples suitable for taxonomic identification (Ricci & Balsamo 2000; Wallace & Ricci 2002) and by the dearth of investigators qualified to make species-level identification of any but a few taxa. In addition, many important dynamics of rotifer ecology may take place over short time spans (Berner-Faukhauser 1987), making traditional microscopic examination of samples impractical. Many of these same difficulties have presented daunting challenges to microbial ecologists, who have responded with a variety of molecular-based techniques to study bacterial and protozoan diversity (see, for example, Amaral Zettler 2002; Polz *et al*. 2003; Spear *et al*. 2003). Two of these approaches, serial analysis of gene tags and microarray hybridization, would seem also to be appropriate to the study of rotifers and other micro-invertebrates.

Serial Analysis of Gene Tags (SAGT)

A number of techniques have been developed to indirectly survey DNA sequence variation in ecological samples, such as rapid amplification of polymorphic DNA (RAPD), single stranded conformational polymorphism (SSCP), and denaturing gradient gel electrophoresis (DGGE). Although these methods have been used to great benefit in the examination of rotifer populations (Gomez *et al*. 2002; Doos *et al.*, this volume;

Papakostas *et al*., this volume), they can be difficult to reproduce, lack resolution, and provide a limited amount of information. In contrast, DNA sequences are highly reproducible, specific, easily compared across studies, and provide much greater resolution of underlying biological processes. However, despite drastic reductions in cost, sequencing remains expensive and, for many studies of ecological samples, inefficient: a single sequencing reaction often provides more information about an individual than is necessary to determine the diversity and relative abundance of taxa in an ecological sample. Serial analysis of gene tags (SAGT) is a new sequencing approach that minimizes the cost of sequencing an ecological sample by maximizing the amount of information about a sample that can be obtained from a single sequencing reaction. This is achieved by combining short stretches of DNA from multiple representatives in a sample and sequencing the concatemer, so that a single sequencing reaction provides information about more than one individual (Kysela *et al*., in press).

The basic SAGT procedure is outlined in Figure 1. Short PCR products are generated from genomic regions that have sufficient sequence information to identify taxa of interest. These amplicons are ligated to form concatemers 2-3 kb in length and the concatemers are then cloned and sequenced. In order to maximize the amount of information obtained from each sequencing reaction, most of the PCR primer sequence is removed prior to ligation by digestion with a type IIS restriction enzyme, which recognizes a sequence incorporated into the 5' end of each primer and cuts ~14 bp upstream of this sequence. This step increases the information in each clone by as much as 30%, and provides cohesive ends for ligation. To maximize the efficiency of ligation, the 5' end of

each primer is biotinylated so that undigested amplicons and 5' digest fragments can be removed.

The utility of SAGT has been demonstrated in a survey of microbial diversity at the hydrothermically active Guaymas Basin in the Gulf of California (Kysela *et al.*, in press). Primers spanning a ~67 bp highly variable region of the bacterial SSU rDNA gene were used to amplify DNA extracted from a sediment sample. Twenty-one of the 35 primer bases were removed by digestion with *Bsg*I, which recognized a restriction site incorporated into the primers. The resulting ~80 bp fragments were concatemerized, cloned, and sequenced. Four to six fragments were read from each sequencing reaction, and more than 90% of the fragments could be identified based on BLAST scores. The bacterial diversity detected was similar to a previous study (Teske *et al.* 2002) of the same sample in which each clone sequenced contained a single PCR product from a single organism, but achieved five times the sensitivity at an equivalent cost.

Microarray Hybridization

The use of microarrays allows the identification of organisms in an ecological sample by hybridizing DNA extracted from the sample to a set of known DNA specimens arrayed on a solid support, usually a microscope slide (reviewed in Call *et al.* 2003). The sample DNA, or more usually PCR product derived from it, is labeled with a fluorophore so that the specific sites of hybridization on the microarray can be visualized and the intensity of the hybridization signal quantified. The method is conceptually identical to Southern hybridization of a dot blot, but technological innovations allow thousands of known DNA

samples to be arrayed on a single microscope slide and many such slides to be produced in a single batch at low cost (Figure 2).

Microarray hybridization has been used to assess microbial diversity and to assay for specific pathogenic bacteria in numerous studies (reviewed in Ye *et al*. 2001; Call *et al.* 2003). Here we demonstrate the applicability of this method to the identification of rotifers.

Materials and Methods

Construction of a rotifer microarray

The rotifer species represented on the microarray are listed in Table 1. DNA for spotting onto the microarray was generated by polymerase chain reaction (PCR) amplification of previously cloned and characterized fragments of the gene encoding the 82-kD heat shock protein, *hsp82*, as previously described (Mark Welch 2000; Mark Welch & Meselson 2000). Sequences, primers, and protocols are available at WheelBase (http://jbpc.mbl.edu/wheelbase). The ~930 bp amplification products were gel purified and their concentrations estimated by spectrophotometery. Aliquots containing 0.5×10^{-6} and 1.0×10^{-6} g of the amplification products were put into the wells of a microtitre plate, the liquid evaporated under vacuum with moderate heat, and the DNA resuspended at 50 or 100 mg/L in 10 microliters of a 50% solution of dimethyl sulfoxide. The DNA solution was spotted onto Corning GAPS II slides using the GeneMachines OmniGrid machine. Slides were UV-crosslinked with 0.3 Joules in a UV Stratalinker 2400 (Stratagene) and were stored over desiccant at room temperature.

Microarray hybridization and visualization

Nick-translation was used to incorporate Alexa 647-12-OBEA-dCTP (Molecular Probes) into 2 micrograms of the purified *P. roseola hsp82*-1 amplification product following standard protocols (Sambrook et al 1989) and using Alexa 647-12-OBEA-dCTP and unlabeled dCTP in a ratio of 1:1. The average length of labeled probe fragment was 200-400 nucleotides. Labeled DNA was separated from unincorporated dNTPs by passage through a G50-150 Sephadex (Sigma) spin column, precipitated with 10×10^{-6} g of blocking DNA (sheared salmon sperm DNA, Eppendorf) and resuspended in 0.2 ml hybridization solution (4x SSC pH 7.0, 0.1% SDS, 50% deionized formamide, 0.14 g/L sheared salmon sperm DNA and 0.2 g/L tRNA).

Microarrays were denatured in deionized water at 95°C for 2 minutes, dehydrated in 95% ethanol for 2 minutes at room temperature, and dried using compressed air. Microarrays were rehydrated by soaking in prehybridization solution (5x SSC pH 7, 0.1% SDS, 1% fraction V BSA) for 45-90 minutes at 42°C, then were washed in deionized water twice for 4-5 seconds each, in isopropanol once for 3 to 4 seconds, and dried by briefly spinning in a centrifuge (Beckman-Coulter TS-5.1-500 rotor at 45 rcf for 2 minutes). Labeled DNA was denatured at 95°C for 3 minutes, cooled on ice, and 20 microliters was applied to each microarray slide. Microarrays and labeled DNA were then covered with Hybri-Slip cover slips (Molecular Probes), placed in hybridization chambers (Corning) and hybridized for 18-23 hours at 42°C.

Microarray slides were washed in 2x SSC, 0.1% SDS at 42°C for 5 minutes, then in 0.1x SSC, 0.1% SDS once for 10 minutes at room temperature (low stringency), or four

times for 5 minutes each at 60°C (high stringency). Slides were then washed three times for 1 minute each at room temperature in 0.1x SSC, immediately dried by briefly spinning in a centrifuge, and stored in a dark box. Hybridization of labeled DNA to the micoarray was visualized using a Gene-Pix 4000 B scanner (Axon Instruments) and GenePix Pro 4.0 software.

Results and Discussion

We printed a microarray of *hsp82* sequences from diverse bdelloid and monogonont rotifers onto each of twelve slides. Two slides were hybridized with fluorescently labeled DNA from *P. roseola hsp82* copy 1 (Pr1); one was washed under low stringency conditions which allow some degree of hybridization between mismatched sequences to remain; the other was washed at a higher stringency which should denature all but the best-paired DNA duplexes. Scanned images of the slides are shown in Figure 3. All sequences other than Pr2 differ from Pr1 by >13%, and under the hybridization conditions used here Pr1 did not hybridize to them at significant levels (1-10% of the hybridization of Pr1 to itself). Pr1 and Pr2 differ by 0.6%, and the average hybridization of Pr1 to Pr2, under conditions of low and high stringency, respectively, was 92% and 78% that of Pr1 to itself.

Our results demonstrate that hybridization of an exact match between labeled DNA and DNA on the microarray is measurably more intense than hybridization of two sequences that differ by as little as 0.6%. In the case of *hsp82*, this would allow sub-species level discrimination within Brachionus, sub-genus level discrimination within

Adineta and sub-family identification within Philodinidae. The use of sequences shown to be evolving more rapidly in rotifers, such as the ITS region of monogononts or the mitochondrial cytochrome oxidase gene of bdelloids (E. Walsh, pers. comm.; Birky *et al.*, this volume), would allow even greater sensitivity. The cross-hybridization of related sequences, particularly under lower stringency conditions, may also be useful, as it can be used to detect the presence of sequences in the ecological sample that are not present on the microarray but are related to those that are.

Ecological samples will likely contain DNA from a mixture of species, at different concentrations depending on relative species abundance. We mimicked such a sample by mixing Pr1 and Hc1 PCR products in a ratio of 1:5, then amplifying and labeling the mixture as described above. We performed five hybridizations using decreasing concentrations of labeled DNA (from 200ng to 0.1 ng) and high stringency washes, which should have produced conditions in which the labeled DNA was limiting in at least some of the hybridizations. While hybridization signal to both Pr and Hc sequences on the microarray decreased with decreasing concentration of labeled DNA, the ratio of the hybridization signal of Pr1 to that of Hc1 never significantly differed from 1:1 (not shown). This suggests that the hybridization reactions in which the labeled DNA was limiting did not reach equilibrium, or that the amplification of the Pr1-Hc1 mixture was significantly biased (Schmalenberger *et al*. 2001). The use of unamplified total ecological sample DNA in the hybridization reaction avoids this latter problem, and has been used with some success with the smaller, less complex genomes of eubacteria (Small *et al*. 2001; Wu *et al*. 2001; Chandler et al. 2003). In preliminary experiments, hybridization of labeled rotifer genomic

DNA was detectable only to very long (~40 kb) DNA fragments on the microarray (not shown), which were available only because of the existence of previously isolated cosmid clones (Mark Welch *et al*., submitted). The use of total genomic DNA on a microarray is complicated by the difficulty of obtaining sufficient amounts for most rotifer species, and in any case the interpretation of hybridization of total genomic ecological sample DNA to a microarray of total genomic DNA would be confounded by a number of factors such as simple repeat DNA and horizontally-transferred transposable elements. However, signal amplification and other improvements in technique may allow for quantitative microarray hybridization in the future (Belosludtsev *et al*. 2001; Small *et al*. 2001; Karsten *et al.* 2002).

A standard application of microarray hybridization in studies of genomic transcription levels involves competitive hybridization of two differentially labeled samples of cDNA to the same microarray slide (Schena *et al*. 1996; Bowtell 1999). This allows quantitative differentiation between transcription levels in the two samples when the relative difference in abundance is greater than about two-fold (Quackenbush 2002). Using the same technique, it should be possible to quantitate >2-fold differences in species abundance between two ecological samples. This would allow rapid and sensitive examination of differences in species abundance in diurnal cycling, for example.

A method that does appear to be reasonably quantitative, and extremely sensitive, is SAGT. Unlike microarray hybridization, SAGT can be implemented by any research group currently using traditional sequencing. While SAGT was developed to allow high-throughput sequencing facilities to generate even more data, the technique may be even more relevant to researchers who are limited in the number of sequencing reactions they

are able to run. Currently, SAGT can produce more than ten sequence tags per sequencing reaction (C. Palacios, personal communication), providing most of the power of full-length sequencing of ecological samples at a fraction of the cost. The use of primers specific for rotifers or specific sub-taxa would allow rapid quantitative surveys of new localities, or the same locality over time, and would be particularly well-suited to the analysis of sediment core samples and resting egg banks. The positive identification of sequence tags generated from a rotifer SAGT experiment is currently limited by the small amount of sequence information available. However, SAGT surveys of little-known microbial flora have been quite successful using bioinformatic and phylogenetic analyses such as BLAST and maximum likelihood to classify tags into known clades (Kysela *et al*., in press). A rotifer-specific example of the success of such a bioinformatically-driven approach can be seen in a survey of the 18S sequences obtained from a sample taken from a cryoconite hole in Antarctica (Christner *et al*. 2003). Christner and colleagues sequenced full-length PCR clones and were able to positively identify a sequence as coming from a Philodina based on BLAST scores alone, despite the fact that only two bdelloid 18S sequences were available in their data base search.

Both SAGT and microarray hybridization are powerful techniques that can greatly expand the amount of information obtained from traditional ecological sampling, potentially drastically increasing the scope and resolution of ecological studies. While both approaches are simple enough that they can be pursued by researchers working independently, their real power will come from collaborative efforts between biochemists, ecologists, geneticists, systematicists, and others, as well as between groups working on

different issues but united in the use of a common study organism. The traditional geniality among those who study rotifer biology makes this field well-suited to benefit from such possibilities. To this end, detailed protocols for microarray hybridization and a list of specific genes currently available for production of a rotifer chip are available on WheelBase (http://jbpc.mbl.edu/wheelbase), the online data base of rotifer biology. Once DNA is available, a microarray can be produced in large quantities at relatively little cost, and the use of indirect detection using such as streptavidin or antibodies can reduce the cost of the hybridization reaction (Alexandre *et al*. 2001). Microarrays may be made for specialized applications, such as discriminating between members of the *plicatilis* group, or for general surveys of rotifers; however, as a single microarray can easily contain a sequence from every known rotifer species (or even multiple sequences from each species), a reasonable goal would be a microarray containing all known rotifer sequences, produced in quantities sufficient for use throughout the community. As new sequences became available, they could be added to new versions of the rotifer microarray chip at regular intervals.

Acknowledgments

We are grateful to Carmen Palacios for discussions of SAGT protocols, to CP, David Kysela, and Mitch Sogin for sharing unpublished SAGT results, and to Steve Biller and Jillian Ward for help in creation of the microarray. This work was supported by the United States National Science Foundation.

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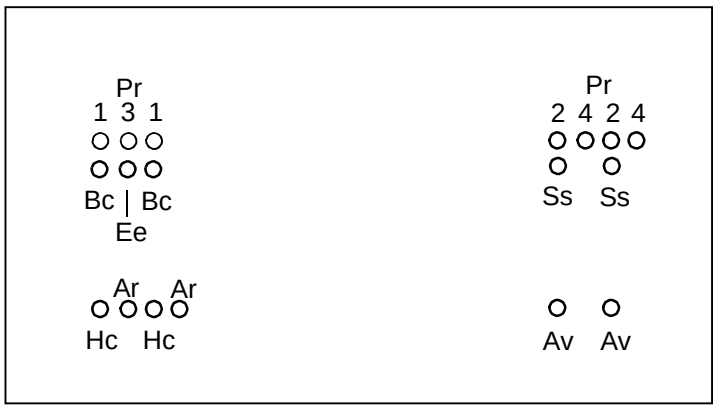
Table 1. Taxa, sequences, and accession codes

Rotifer *hsp82* sequences	GenBank accession	% difference from *P. roseola* copy 1
Bdelloidea		
Adineta vaga (Davis, 1873) copy 1	AF143849	13.5
Adineta ricciae Segers 2003 copy 1	XXXXXX	15.2
Habrotrocha constricta (Dujardin, 1841) copy 1	AF143850	15.1
Philodina roseola Ehrenberg, 1832 copy 1	AF143851	—
P. roseola copy 2	AF249997	0.6
P. roseola copy 3	AF250002	13.1
P. roseola copy 4	AF250004	13.4
Monogononta		
Brachionus calyciflorus Pallas, 1766	AF143855	25.0
Eosphora ehrenbergi Weber, 1918	AF143858	22.3
Sinantherina socialis (Linnaeus, 1758)	AF143854	25.0

Figure 1. Outline of SAGT. In traditional sequencing surveys (left), DNA from a sample (a) is amplified (b), cloned and sequenced (c). In SAGT (right) shorter amplification products (b) are created; each product (b1) consists of primers (grey bars) that are 5' biotinylated (B) and contain a recognition sequence for a type IIS endonuclease (small black bars), which digests the primers 10-20 bases 3' of the recognition sequence (arrows) to remove most of the primer sequence and create 3' overhangs (b2). The DNA is passed though a streptavidin column to remove the biotinylated primer fragments and undigested PCR product, and the remaining fragments are ligated together (b3). These concatemers are cloned and sequenced (c); thus a single sequencing reaction of a single clone yields information about multiple original PCR products.

Figure 2. Outline of microarray hybridization. DNA from an ecological sample (a) is amplified and the amplification products (b) labeled with a fluorophore (alternatively, total DNA may be labeled). Labeled DNA (c) is used to probe previously characterized DNA immobilized in spots on a microscope slide (often called a chip, d). Spots of known DNA to which the fluorescently labeled probe DNA has hybridized can then be visualized (e).

Figure 3. Hybridization to a rotifer microarray. Top, schematic showing the location of rotifer DNA on the chip, designated by genus and species initials; middle, hybridization after low stringency washing; bottom, hybridization after high stringency washing. Only portions of the microarray slides are shown.



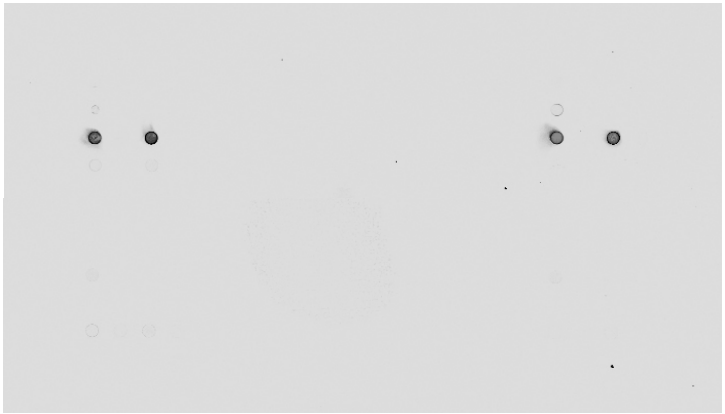
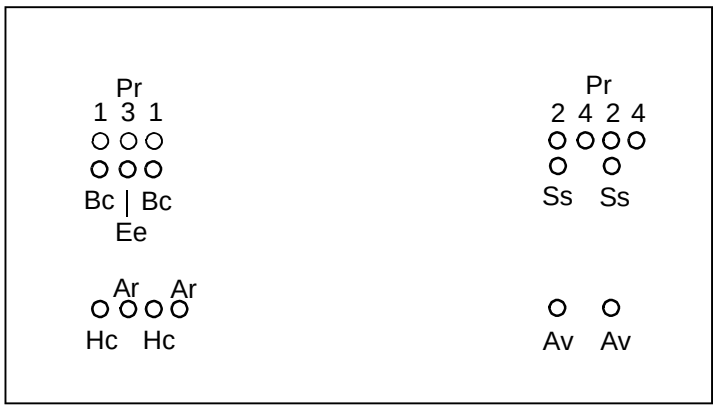


Figure 3



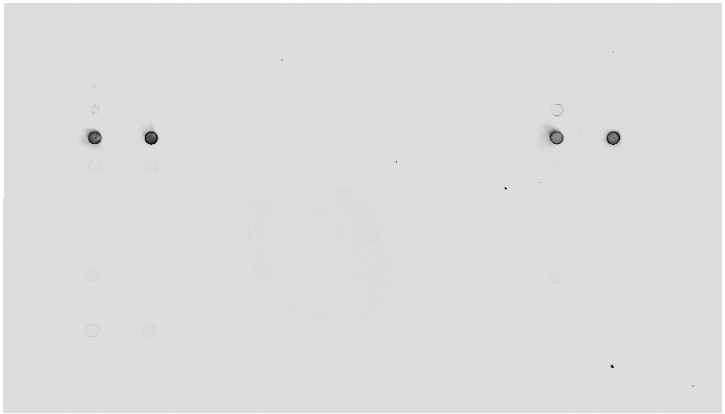


Figure 3

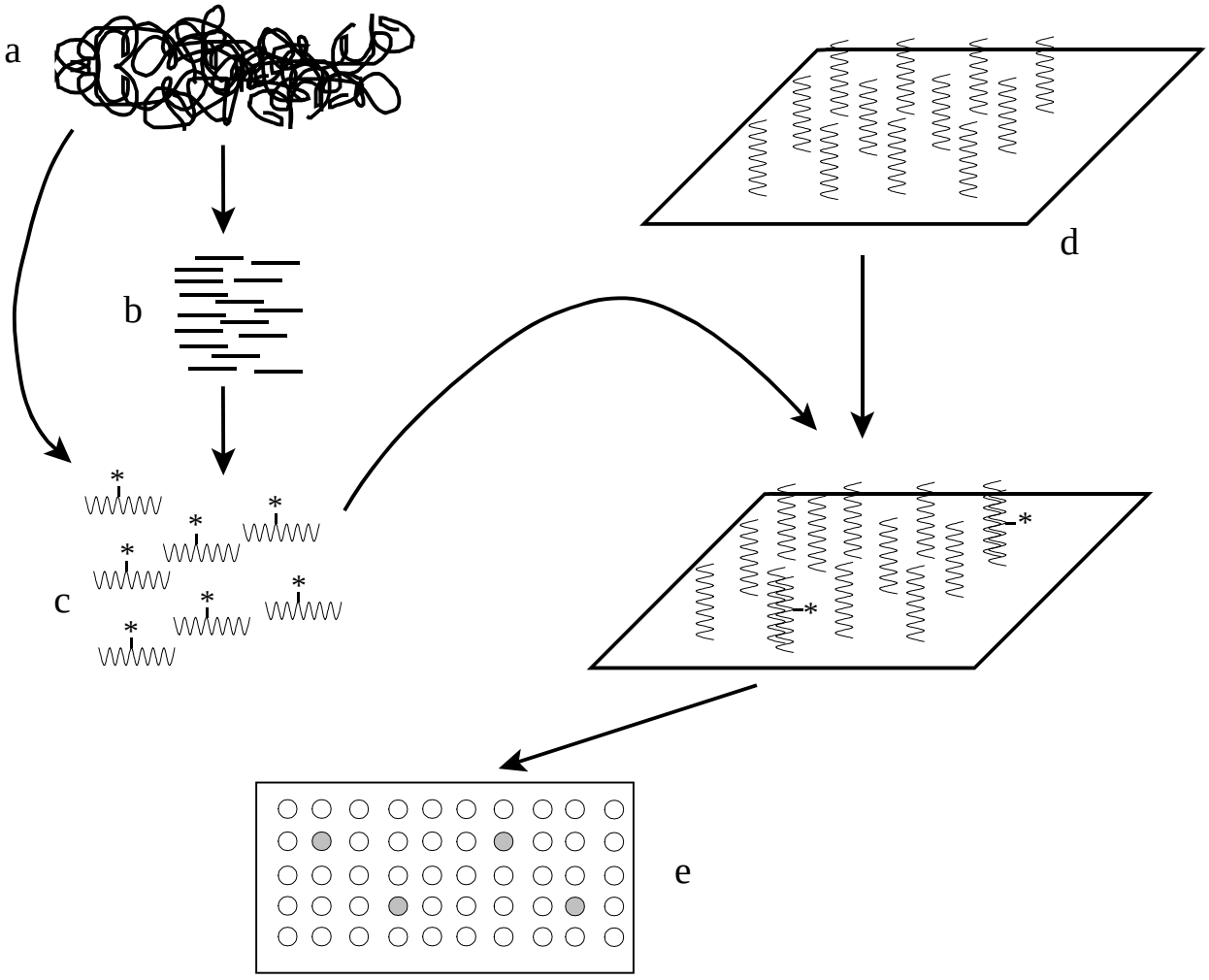
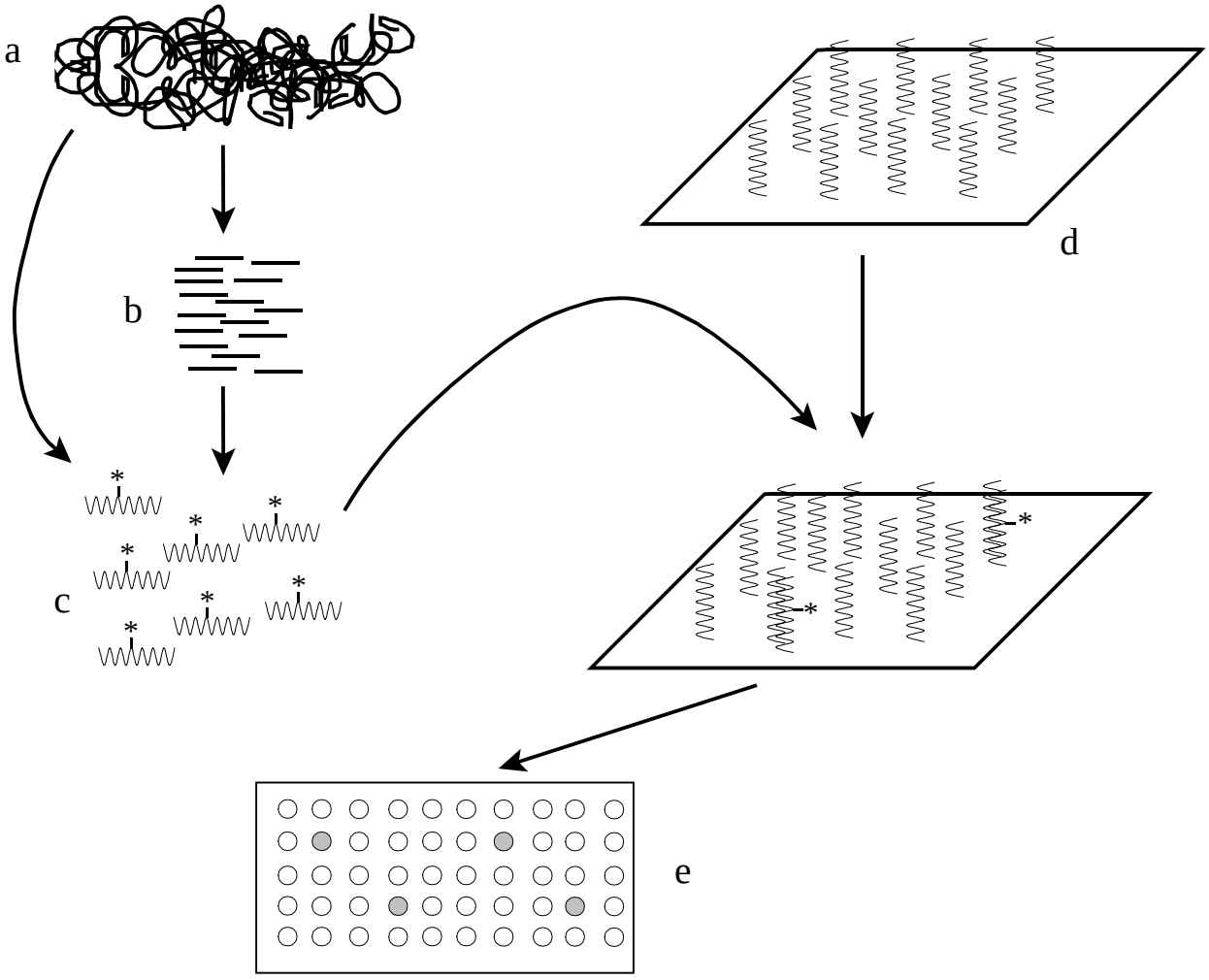


Figure 2



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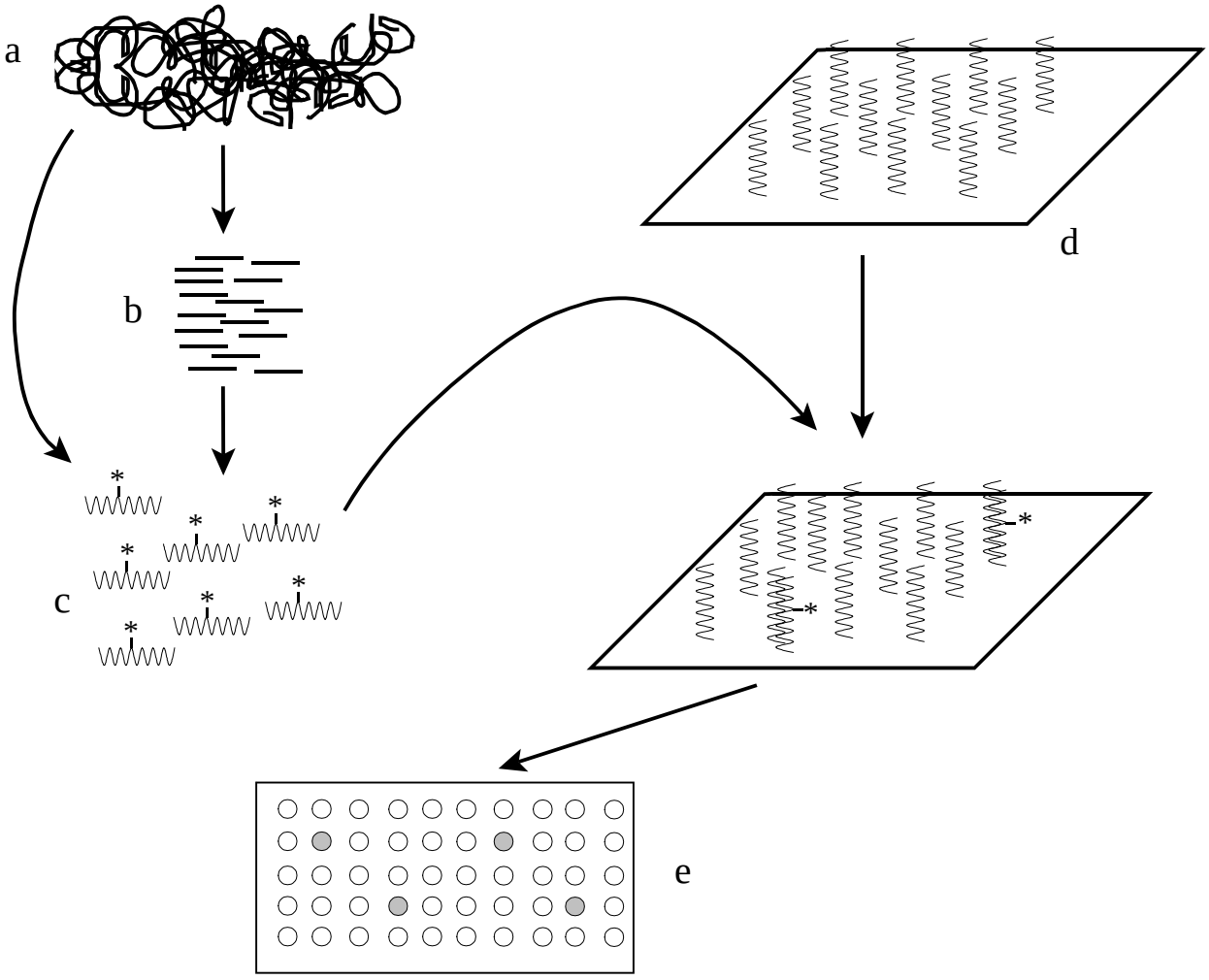

Figure 2

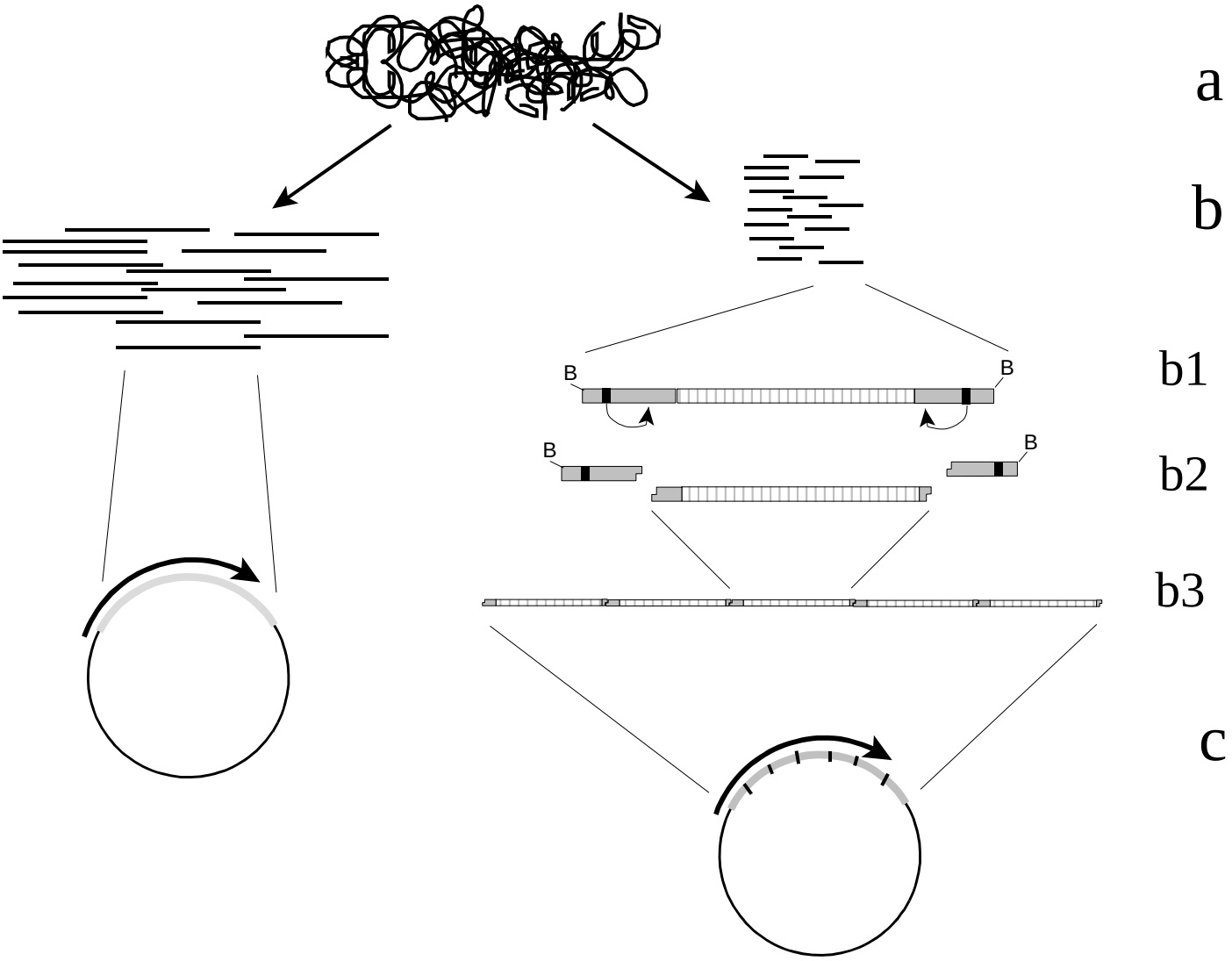

Figure 1

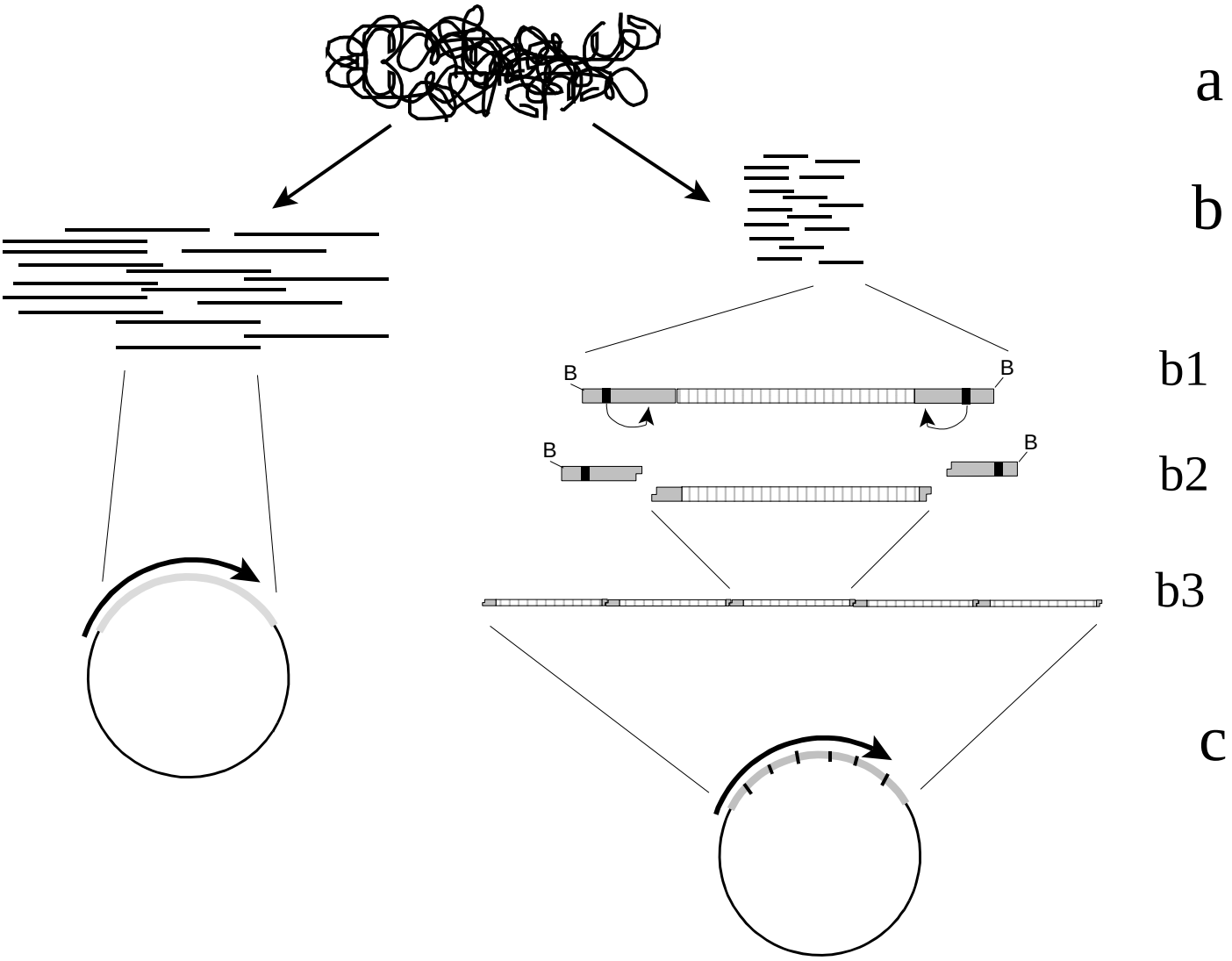

Figure 1

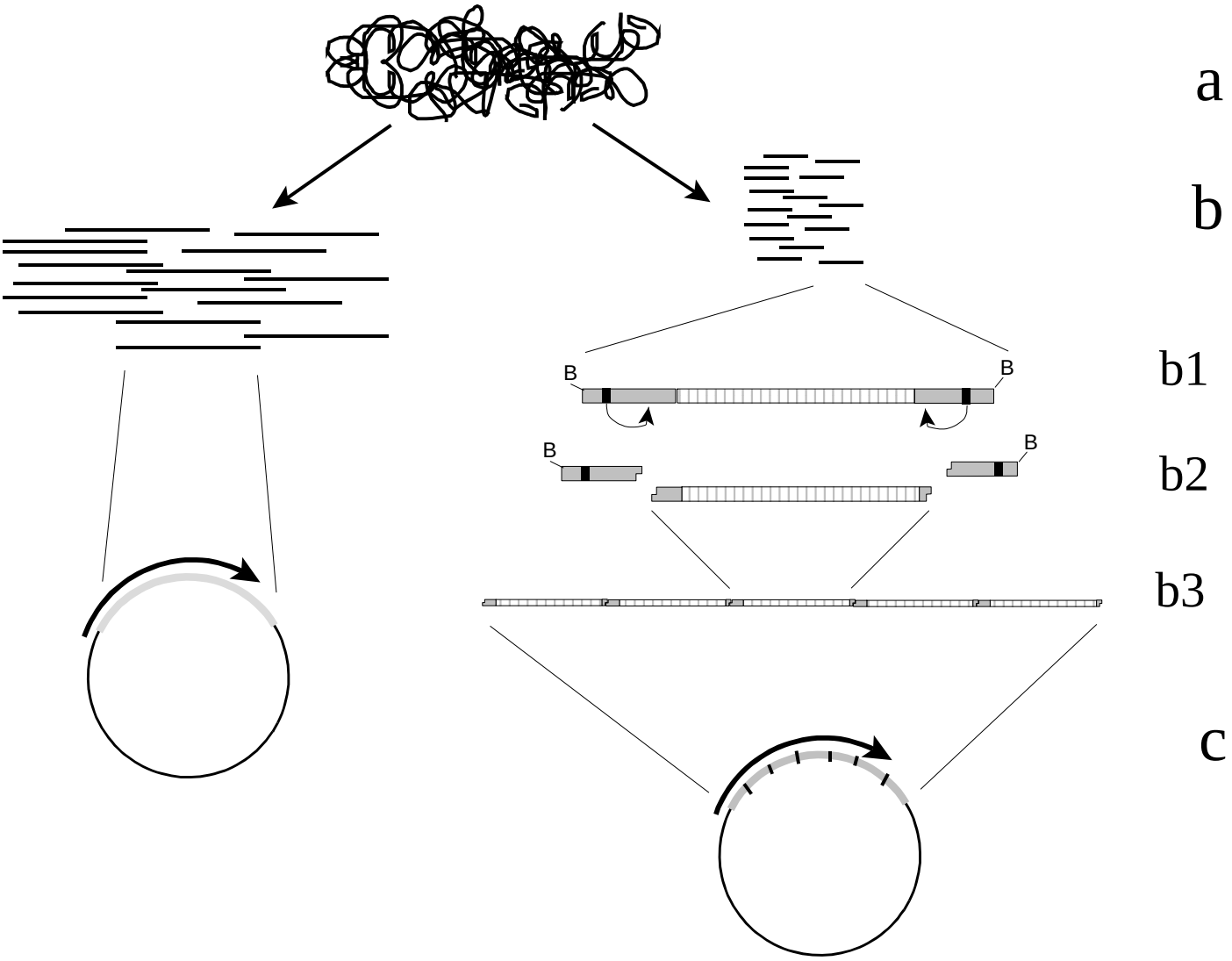

Figure 1