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Applicant: INTERNATIONAL UNIVERSITY BREMEN GMBH
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High throughput electropermeation system

The present invention relates to an apparatus and a method for high throughput electropermeation, particularly of fungus cells. Particularly, the invention relates to multi-well arrays for electropermeation of cells and for extracting proteins and, in particular, nucleic acids from cell samples.

Electropermeation, as understood in the scope of the present invention, is a technique for introducing reversible or irreversible pores into the cell membrane and optionally to the cell wall of a cell by application of one or more electrical pulses. Electropermeation is thus related to the general art of electroporation, which is understood to be the technique of disrupting cells and/or cell components by application of electrical field.

It is frequently necessary to extract cell constituents from a cell sample. Particularly, when screening a cloning library, it is necessary to analyse which of the respective clones harbours a protein or nucleic acid of interest.

For extraction of cell contents, a variety of techniques has so far being established.

One of these techniques is sonication. In sonication, mechanical energy in the form of ultrasound waves is introduced into a cell sample. The cells of the cell sample are disrupted by the shear forces of the ultrasound waves, thus releasing the cell constituents into a surrounding medium. However, sonication does not yield good results on many different cell types. Also, the amount of mechanical energy introduced to disrupt and lyse a cell sample commonly leads to a considerable heating of the cell sample, damaging or destroying sensitive and valuable cell constituents.

A technique mainly used for introducing nucleic acids into cells is electroporation. In electroporation, a short pulse of electrical energy is applied to a cell sample. The electrical pulse leads to an uptake of charged molecules from a surrounding medium into the electroporated cells. A fraction of cells is also destroyed, thereby liberating their constituents into the surrounding medium.

The ability to release cell constituents from a cell sample by electroporation has been exploited in a number of patent applications. For instance, WO 98/54306 discloses a method for isolating an intracellular substance, whereby whole cells of a cell suspension are lysed by application of an electrical pulse. From the cellular detritus, nucleic acids and proteins of interest are then separated. However, with the method of the aforementioned international patent application it is not possible to extract nucleic acids and proteins gently. In particular, nucleic acids will often be destroyed by the action of nucleases that are released from the lysed cells. Similar electroporation methods are described in WO 00/63355.

For the first time Ganeva and Galutzov (Electropulsation as an alternative method for protein extraction from yeast. FEMS Microbiology Letters 174 (2):279-284, 1999) have demonstrated that it is possible to extract proteins from yeast in batch without destroying of intracellular organells. Finally in WO 0233065 the optimal conditions for protein production by electroporation has been described. The invention concerns a method for producing proteins which consists in subjecting a liquid medium flow comprising at least a yeast, bacterium or cell of mammals to electroporation, and recuperating the released proteins. The method comprises an electroporation step followed by an incubation step.

From WO 2005/042696 A2 and WO 2005/044983 A2 are known apparatuses for electroporation treatment of cell samples in a conventional 96 well array. However, these patent applications also do not disclose a method for gentle extraction particularly of nucleic acids, but also of proteins. There is still an immanent danger of destruction of sensitive cell constituents (particularly nucleic acids) by the release of digesting enzymes, particularly nucleases.

In high throughput applications, it is necessary, to achieve a desired goal (e.g. extraction of nucleic acids and/or proteins) with a minimum amount of work. Normally, high throughput applications are highly automated. It is thus not possible to visually inspect a cell sample for adapting the treatment of that sample to optimal conditions. Methods for high throughput analysis therefore have to work under a variety of conditions, particularly with different cell types like yeasts, gram-positive and gram-negative bacteria, plant cells and mammalian cells. They also have to provide a good product yield with very few steps. The method steps should only comprise very basic operations and should not rely on elaborate apparatuses. For example, while a centrifugation is viable also in high throughput applications, other techniques like, for example, phenol-chloroform-extraction are to be avoided because they require frequent change of media and much visual inspection of treated cell samples. Particularly cell constituent extraction methods must therefore provide the desired cell constituent in a high yield and high purity.

It was therefore a problem of the present invention to provide an apparatus and a method for high throughput extraction of cell constituents, particularly of nucleic acids and/or proteins.

According to the invention, there is thus provided a high throughput electropermeation system, comprising

- a pair of electrodes spaced apart from one another, for applying an electric voltage to a cell sample for electropermeating cells of the cell sample, and

- a control unit for controlling the voltage applied through said pair of electrodes.

Earlier research (WO 02/33065) has shown that electropermeation results in the formation of irreversible pores in a cell membrane and optionally also in a cell wall, which leads to the release of cytoplasmic proteins in high purity, while avoiding the release of potential destructive enzymes from cell organelles, particularly from vacuoles. Current research additionally has shown the liberation of plasmids from electropermeated cells. Therefore, electropermeation is particularly suitable for high throughput analysis applications, as it allows simultaneous releasing of nucleic acids, particularly plasmids, and water-soluble proteins in high purity from cells of a cell sample. The method avoids cumbersome extraction steps as, for instance, necessitated by phenol-chloroform-extraction, and is readily scalable to simultaneously treat a vast number of cell samples. Further benefits of the high throughput electropermeation system and method of the invention will be described below.

The high throughput electropermeation system comprises a pair of electrodes spaced apart from one another. The electrodes are for applying an electric voltage to a cell sample. The electric voltage applied will cause an electric current to flow through the cell sample, partially disrupting a cell wall in a cell sample to cause the formation of pores. Through such pores, cell contents leak to the outside medium. By appropriately adjusting the voltage, electrical field and pulse frequency, the skilled person can prevent the rupture of further cell organelles and can thus achieve a liberation of cytoplasmic cell constituents in high purity. The inventors have found that high throughput electropermeation is particularly well suited for extraction of plasmids from cells. By a simple centrifugation of the electropermeated cell sample and collection of the supernatant, nucleic acids and particularly plasmids of a cloning library can be harvested and directly used for high throughput nucleic acid amplification, particularly high throughput PCR.

The high throughput electropermeation system of the present invention also comprises a control unit for controlling the voltage applied through said pair of electrodes. The control unit adjusts the electrical field such that the cell sample is treated by electropermeation, whereby pores in the cell membrane are

irreversibly formed. It is particularly preferred that the control unit has further functions as described below.

The high throughput electropermeation system of the present invention is particularly suitable for electropermeation of yeasts, gram-negative bacteria and mammalian cells. It is particularly preferred when the cell sample to be electropermeated is a yeast cell sample. Preferably, the cell sample used for electropermeation is selected from cells of the species *Saccharomyces cerevisiae*, preferably *Saccharomyces cerevisiae PV3* (diploid), *Saccharomyces cerevisiae Y47* (wild haploid), *Saccharomyces cerevisiae SPY509* and *Saccharomyces cerevisiae InvSC*, *Kluyveromyces lactis*, and *Schizosaccharomyces pombe*. It is also preferred that the cell sample is a sample of gram-negative bacterial cells, preferably *Escherichia coli*. If the cell sample is a sample of mammalian cells, it is particularly preferred that the cell sample comprises fibroblast cells, in particular Chinese hamster cells. All of the aforementioned cell types are particularly suitable for high throughput screening applications. It is particularly advantages that the high throughput electropermeation system of the present invention allows for the extraction of nucleic acids and proteins from yeast cells, as such cells notoriously release nucleases, thus conventionally necessitating considerable effort for extracting nucleic acids. It is believed that the present invention avoids the release of large quantities of nucleases by spearing the yeast cell vacuole from disruption.

The high throughput permeation system of the present invention preferably comprises a plurality of wells, each for containing a cell sample to be electropermeated. The wells are preferably arranged in a standard high throughput array format. Particularly, the wells can be arranged in a conventional 96 well array with a total of 12 columns, each comprising 8 wells. Such arrays and suitable arrays of electrodes are described in WO 2005/042696 A2 and WO 2005/044983 A2, which are incorporated herein by reference.

It is likewise preferred that the high throughput electropermeation system of the present invention comprises an array of electrode pairs conforming to an array of wells for containing cell samples to be treated by electropermeation. This way, for each cell sample an individual pair of electrodes can be assigned, thus limiting

the risk of contamination by carrying minute quantities of one cell sample from one well into the cell sample of another well, if one electrode were assigned to two or more cell samples. It is particularly preferred that the high throughput electropermeation system of the present invention comprises a number of electrode pairs suitable for treating simultaneously one row or one column of the cell sample well array. For instance, the high throughput electropermeation system of the present invention may comprise a column of eight electrode pairs for treating eight wells of a column of a conventional 96 well microtitre plate ("microplate"). It is likewise preferred that the high throughput electropermeation system of the present invention comprises a row of 12 electrode pairs for treating a row of 12 vales of a conventional 96 well microplates. For other cell sample well arrays, a corresponding number of other electrode pair configurations can be chosen. However, it is particularly preferred to provide a number of electrode pairs equal to the number of wells comprised in the cell sample array to be treated by electropermeation. It is thus particularly preferred to provide 96 electrode pairs for treating cell samples provided in a conventional 96 well microplate array.

Furthermore, it is particularly preferred that the pair of electrodes or all pairs of electrodes of the high throughput electropermeation system of the present invention conforms to the side walls of the well or wells, respectively, comprising the cell sample(s) to be treated. This way, the pair of electrodes displaces the cell sample and forces the cell sample into the gap between the electrode pairs, thereby securing a uniformly applied electrical field on the cell sample.

For electropermeation, it is preferred that the control unit of the high throughput electropermeation system according to the present invention is configured to provide an electrical field intensity of at least 3 kV/cm at the pair of electrodes for electropermeating a cell sample. If the high throughput electropermeation system according to the present invention comprises more than one pair of electrodes, it is preferred that the electrical field intensity of at least 3 kV/cm can be applied at all pairs of electrodes. It has been found that the onset of electropermeation can be well described by the electrical field applied to a cell sample more or less regardless of the type of cells to be electropermeated. Thus, even though there are differences in membrane composition for cells of different types, particularly

for the preferred cell types mentioned above, it appears that all cell types commonly used in research can be electropermeated by the application of the same electrical field intensity. It is also preferred that the electrical field intensity is limited to 5 kV/cm, the electrical field intensity of 3,5 - 4 kV/cm and particularly 3,75 kV/cm being especially preferred, particularly for electropermeation of the aforementioned yeast cell types.

The or each pair of electrodes of the high throughput electropermeation system according to the present invention comprises a gap between the electrodes of at most 3 mm, more preferably at most 2 mm. An electrode gap of 2 mm has been found to be particularly well suited for treating cells in a conventional 96 well microplate array.

The electrodes of the high throughput electropermeation system according to the present invention are substantially parallel to each other. The electrodes are of a conductive, durable and inert material that does not, for example, release ions interfering with electropermeation and the liberation of cell contents. Preferred electrode materials are aluminium, gold and platinum. Stainless steel electrodes have been found to be less durable than electrodes of the preferred electrode materials, but can also be used.

Also preferably, the electrodes are formed on a non-conductive base member. The base member is preferably made from a durable, water resistant plastic, preferably from conventional circuit board stock. The electrodes can be bonded to the non-conductive base member by conventional techniques, e.g. by mechanical or chemical securing of the electrodes on the non-conductive base member. Preferably, the electrodes including the respective non-conductive base member are resistant against sterilisation events and particularly resistant against heating to 200°C. It is particularly preferred that the pair(s) of electrodes of the present invention is sterilised before being used for electropermeation of a respective cell sample.

The control unit of the high throughput electropermeation system according to the present invention is preferably configured to provide, for electropermeating a cell sample, one or more electrical pulses with an electrical field intensity of 3 - 5

kV/cm, preferably 3,5 - 4 kV/cm and more preferably of 3,75 kV/cm, and a pulse width of 0,1 to 100 ms, preferably 0,5 to 15 ms, more preferably 1 to 5 ms and most preferably 1,5 ms. The exact electropermeation conditions can depend on the cell type to be treated; generally, the aforementioned most preferred conditions are for treating yeast cells, particularly *Saccharomyces cerevisiae* cells. The aforementioned pulse characteristics are particularly suitable for avoiding electroporation conditions and large-scale cell disruption.

It is particularly preferred that the control unit of the high throughput electropermeation system according to the present invention is configured to provide, for electropermeating a cell sample, 1 to 100 of the electrical pulses described above, preferably 5 to 20 pulses and more preferably 15 to 20 pulses, at a frequency of 0,1 to 100 per second, preferably 0,1 to 10 per second, and most preferably 0,1 to 2 per second. Best results for electropermeating yeasts, particularly *Saccharomyces cerevisiae*, were obtained by application of 20 pulses at a frequency of one per second with an electrical field intensity of 3,75 kV/cm and a pulse width of 1,5 ms. Under these conditions, particularly high yields of proteins and plasmids could be extracted in a good purity that was suitable for immediate enzyme assay and PCR nucleic acid amplification without first performing an purification step other then collecting the supernatant of the cell sample after centrifugation.

The high throughput electropermeation system and corresponding method according to the present invention is easy to operate, the method is fast and does not necessitate laborious sample preparation. The electropermeation system of the present invention has very low running costs and is scaleable to high throughput screening applications.

By using electrical pulses as defined above, it is possible to achieve extraction of nucleic acids and proteins, particularly enzymes, without major cell decompositions. Thus, the release of balk proteins, lipids, genomic DNA and cell debris can be avoided. This is particularly useful for extracting nucleic acids like plasmids, as the extracted plasmids or other nucleic acids can be readily used in further amplification methods, particularly in PCR methods. Also, if proteins and

particularly enzymes are to be extracted, they can be stabilised by using an appropriate stabilisation buffer as medium for the cell sample.

After application of one or more electrical pulses according to the present invention, it is preferred to diminish electrostatic interactions between electropermeated cells on the one hand and the contents - proteins and/or nucleic acids - to be liberated from said cells. It is therefore preferred to perform the electropermeation in a medium with high resistance, preferably in deionised water, and to add a buffer comprising glycerine and dithiothreitol after electropermeation. Suitable buffers and cell sample treatment methods are described in Ganeva et al, FEMS Microbiology Letters 174(2), 279-284, "Electropulsation as an alternative method for protein extraction from yeasts", the contents of which are incorporated herein by reference. It is correspondingly preferred when the high throughput electropermeation system of the present invention further comprises a dispenser for adding glycerine and/or dithiothreitol to a treated cell sample.

The cell contents, particularly proteins and/or nucleic acids, liberated by electropermeation are harvested by separation of the sample's cells from the medium, preferably by centrifugation, and removal of the medium. Correspondingly, a preferred high throughput electropermeation system of the present invention further comprises means for separating the cell sample's medium from the sample cells of the one or more electropermeated samples, wherein the separation means preferably are centrifugation means and suction means for removing a supernatant after centrifugation.

The following examples and figures describe particularly preferred embodiments of the system and methods of the present invention, without limiting the scope of the claims:

Figure 1 is a schematic drawing of a pair of electrodes useful in the high throughput electropermeation system of the present invention; and

Figure 2 is a distribution graph of protein (a) and plasmid (b) extraction efficiency in 94 electropermeation experiments.

Figure 1 shows a schematic representation of a pair of electrodes useful in a high throughput electropermeation system according to the present invention. The pair of electrodes comprises two electrodes 10, 10'. The electrodes 10, 10' are of a conductive, steriliseable and preferably heat-resistant material, preferably aluminium. In particular preferred embodiments of the present invention, the electrodes are of a material that prevents the release of ions and particularly of toxic metal ions to a cell sample. The electrodes 10, 10' have a thickness of 1 mm. They are planar and adjusted in parallel to each other, thereby delimiting a gap 15 of 2 mm.

The electrodes 10, 10' are mounted on a dielectric, non-conductive base member 11, 11'. The non-conductive base member 11, 11' is shaped to conform to the shape of a well of a 96 well microplate. Upon insertion of the pair of electrodes into a corresponding well, the base member 11, 11' and the electrodes 10, 10' work together to displace the cell sample into the inter-electrode gap 15.

Example: High throughput electropermeation of yeast cells

Saccharomyces cerevisiae InvSC1 cells (in vitro gene; genotype: MATα or a, his3delta1 leu2 trp1-289 ura3-52; phenotype: His^-, Leu^-, Trp^-, Ura^-) transformed with plasmid pYES2-GOx (GOx: glucoseoxidase from A*spergillus niger*) were grown in 96 well microtitre plates on selective minimal media at 30°C and 250 rpm. The yeast cells were harvested when they reached an optical density OD_{660} < 2.0. The cells were washed from the growth medium by triple centrifugation at 4.000 g for 1 minute and re-suspension in distilled water. The washed cells were finally re-suspended in distilled water to a concentration of 10 mg of wet cell biomass per ml. The suspension was then electropermeated.

For electropermeation, electrodes of the type described above with reference to figure 1 were employed. A maximum of 500 µl cell suspension per deep-well microtitre plate well (96 wells per microtitre plate) could be electropermeated, as the electrodes forced the cell suspension of the microtitre plate into the gap between the electrodes. The cells were electropermeated by applying 15 to 20 pulses, while each of the pulses has a duration of 1,5 ms and the interval

between the pulses was 1 second. The electrical field strength applied for the pulse was 3,75 kV/cm.

Right after the electropermeation, 100 µl of sixfold concentrated incubation buffer was adder per well to obtain a final buffer concentration of 20 mM phosphate buffer (pH 7,0), 200 mM glycerol, 2 mM $MgCl_2$ and 2 mM dithiothreitol (DTT). The microplates were incubated at 30 °C for one hour at 700 rpmAfter incubation, the medium was harvested by centrifugation at 4.000 g for ten minutes and collection of the cell free supernatant.

Total proteins liberated from the cells were quantified by light adsorption at 280 nm or by NanoOrange protein quantification kit (molecular probes), following the manufacturers instructions. On average, after 1 hour incubation 82.56 µg of total protein was liberated (n=94) per mg of wet yeast biomass. Also, the release of pYES2-GOx was detected by real time PCR (M x 3.000 P real time PCR system, stratagene) using a QPCR SybrGreen kit. The PCR conditions were: initial denaturation for 15 minutes at 95°C, then 40 cycles of: 15 seconds at 95°C, 30 seconds at 55°C, 30 seconds at 72°C. A final denaturation step of 1 minute at 95°C followed. Reaction mixture: 12,5 µl of twofold concentrated Qiagen-Master-Mix, 0,13 µl of forward primer (100 mM), 0,13 µl of reverse primer (100 mM), 7,25 µl water and 5 µl sample. Obtain PCR products were validated by electrophoresis (not shown). Table 1 shows the result of the real time PCR amplifications. Table 2 and corresponding figures 2a and 2b show a statistical analysis of 94 electropermeations in a microtitre plate format.

Table 1

	V_{50} ± SEM	DNA template concentration	Plasmid concentration per mg of wet cell biomass
Positive control – pYESs-GOx	19.08 ± 0.025	3.5 ng μl^{-1}	
EP-extract pure	29.81 ± 0.028	2.061 pg μl^{-1}	247.3 pg mg^{-1}
EP-extract 1 : 10	32.99 ± 0.038	0.227 pg μl^{-1}	
EP-extract 1 : 100	36.48 ± 0.061	0.020 pg μl^{-1}	
Negative control - water	-	-	

SEM – standard error of mean

V_{50} – number of cycles at halfway between bottom and top for the Boltzmann sigmoid

Table 2

	Total protein liberation, [mg ml^{-1}]	Total protein concentration per mg of wet yeast biomass [µg mg^{-1}] (% yielding)	Plasmid liberation, [number of cycles at V_{50}]	Plasmid concentration per mg of wet yeast biomass [pg mg^{-1}]
Mean	0.688	82.56 (12.2%)	29.81	247.3
Standard deviation (*SD*)	0.297		0.373	
Sample size (*N*)	94		94	
Coefficient of variation (%)	43		1.3	
Std. error of mean (*SEM*)	0.033		0.039	
Lower 95% conf. limit	0.623		29.73	
Upper 95% conf. limit	0.753		29.88	
Minimum	0.226		28.51	
Median	0.659		29.81	
Maximum	1.553		30.58	
Normality test KS	0.086		0.067	
Normality test P value	>0.10		>0.10	
Passed normality test?	Yes		Yes	

KS – Kolmogorov-Smirnov normality test

V_{50} – number of cycles at halfway between bottom and top for the Boltzmann sigmoid

Claims

1. High throughput electropermeation system, comprising

- a pair of electrodes spaced apart from one another, for applying an electric voltage to a cell sample for electropermeating cells of the cell sample, and
- a control unit for controlling the voltage applied through said pair of electrodes.

2. High throughput electropermeation system according to claim 1, characterized in that it further comprises a plurality of wells, each for containing a cell sample to be electropermeated.

3. High throughput electropermeation system according to claim 2, characterized in that the pair of electrodes conforms to the side walls of a corresponding well.

4. High throughput electropermeation system according to one of the previous claims, characterized in that the control unit is configured to provide an electrical field intensity of at least 3 kV/cm at the pair of electrodes for electropermeating a cell sample.

5. High throughput electropermeation system according to one of the previous claims, characterized in that the electrodes of the pair of electrodes are spaced apart with a gap of at most 2 mm.

6. High throughput electropermeation system according to one of the previous claims, characterized in that the control unit is configured to provide, for electropermeating a cell sample, one or more electrical pulses with

- an electrical field intensity of 3-5 kV/cm, preferably 3,5-4 kV/cm, and
- a pulse width of 0.1 to 100 ms, preferably 0.5 to 15 ms, most preferably 1 to 5 ms.

7. High throughput electropermeation system according to claim 6, characterized in that the control unit is provided to provide, for electropermeating

a cell sample, 1 to 100 of said pulses, preferably 5 to 20 pulses and most preferably 15 to 20 pulses, at a frequency of 0.1 to 100 per second, preferably 0.1 to 10 per second, and most preferably 0.1 to 2 per second.

8. Method of high-throughput electropermeation, comprising the steps of

a) providing a cell sample in a high-throughput format,

b) inserting a pair of electrodes into the cell sample,

c) applying one or more electrical pulses as defined in claim 6 or 7 to the cell sample through the pair of electrodes.

9. Method of high-throughput extraction of nucleic acids from a cell sample, comprising the steps of

a) providing a cell sample in a high-throughput format,

b) inserting a pair of electrodes into the cell sample,

c) applying one or more electrical pulses as defined in claim 6 or 7 to the cell sample through the pair of electrodes.

10. Use of one or more electrical pulses as defined in claim 6 or 7 for high-throughput electropermeation of a cell sample.

11. Use of one or more electrical pulses as defined in claim 6 or 7 or a high throughput electropermeation system according to one of claims 1 to 7 for high throughput extraction of a protein and/or a nucleic acid from a cell sample.

Abstract

The present invention relates to an apparatus and a method for high throughput cell electropermeation, the apparatus exemplified on fungus cells. Particularly, the invention relates to multi-electrode arrays for electropermeation of cells in microplate and for liberation of proteins and, in particular, nucleic acids from cell samples.

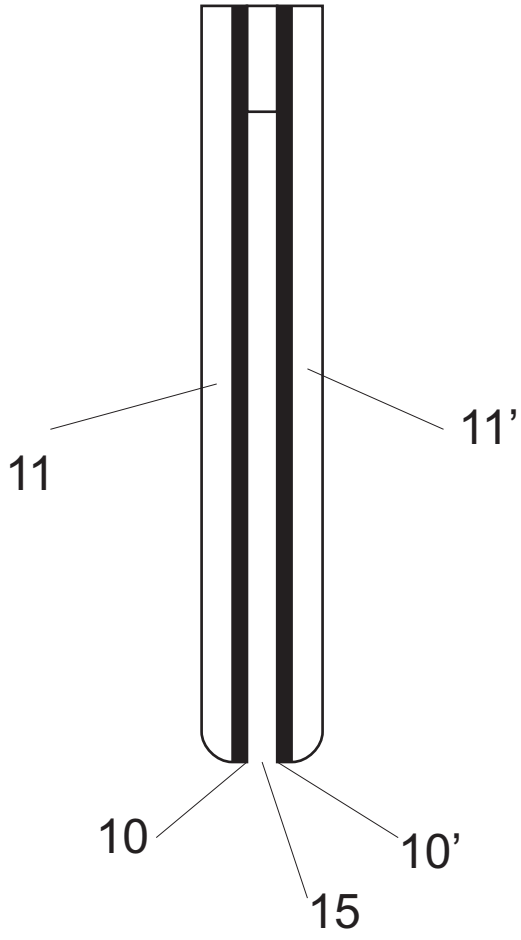

Fig. 1

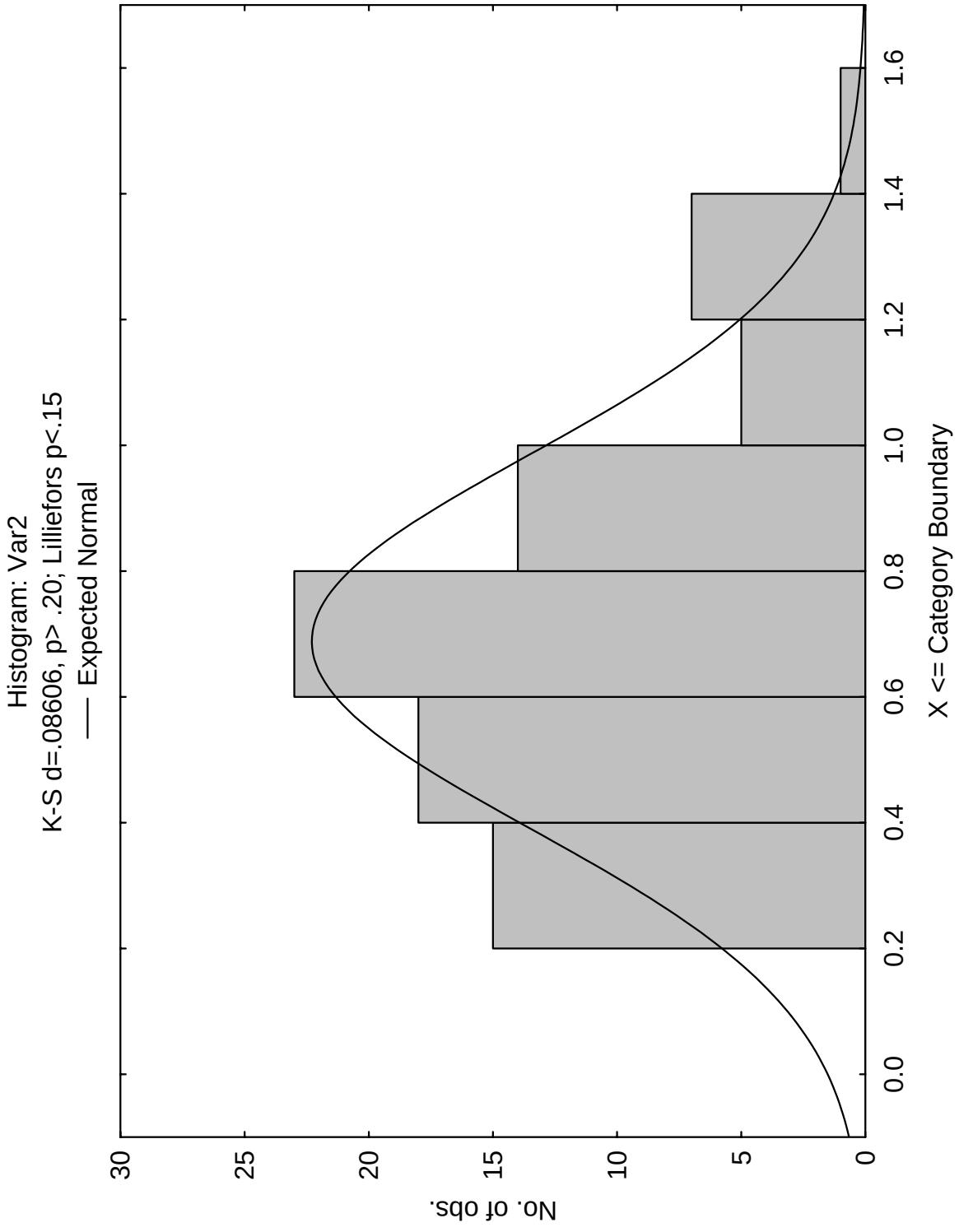
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Fig. 2a

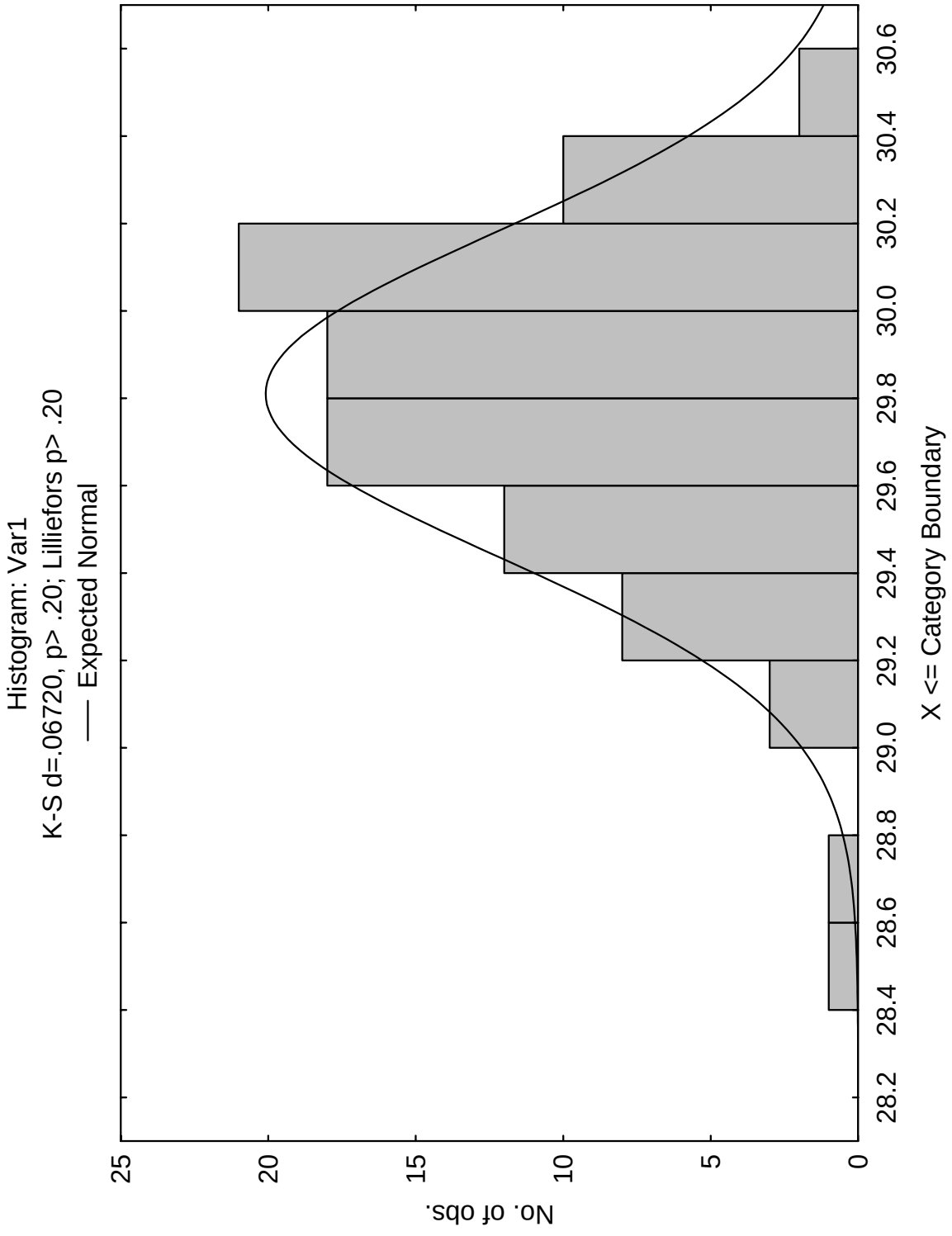
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Fig. 2b