

ENCYCLOPEDIA OF BIOLOGICAL METHODS



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Encyclopedia of Biological Methods

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About this Book

This book is intended for anyone with a good understanding of college-level introductory biology.

This project was born out of Dr. Mattaini's experience developing a Biology of Cancer class in Spring 2021 at Roger Williams University. In the syllabus, Dr. Mattaini revealed her two big-picture goals for the course: "First, I would like you to learn enough basics of cancer biology to be able to independently research and answer your future questions about cancer. Second, I will also support you as you strive to become a more nuanced reader of the primary literature, especially as it relates to cancer biology." The students in the course had all taken an introductory genetics course, but they could be sophomores to seniors, and so had varied levels of experience with the primary literature. To support them in their reading, Dr. Mattaini discovered that it would be useful to have one central and undergraduate-level resource that simply explained the various techniques that the students would encounter while reading the primary literature.

This is a living document that will be updated organically as need arises and time, funding, and collaboration permit. Comments, critiques, suggestions for new content, and welcome offers to collaborate can be directed to the editor by email. [Email: Katie Mattaini](mailto:katie.mattaini@rwu.edu).

About the Editor

Dr. Katherine (Katie) Mattaini is a Lecturer of Biology in the Department of Biology at Tufts University in Medford, MA, USA.

From her undergraduate to postdoctoral work, her research experience has varied widely, from organic synthesis to mouse work. Her focus from her PhD onward was cancer metabolism. This broad background has prepared her well for teaching undergraduates, which she considers her calling in life. During her post-doctoral fellowship, Dr. Mattaini participated in the TEACRS (now Tufts IRACDA) program, gaining experience in pedagogy, teaching, inclusive practices, and other important skills.

Beginning in 2017, Dr. Mattaini has had the joy and privilege of teaching undergraduates full-time, first at the College of the Holy Cross (Worcester, MA), then Roger Williams University (Bristol, RI), and now at her postdoctoral institution, Tufts University (Medford, MA). Over this time, she has taught Introductory Biology lecture and lab, Biochemistry I and II, Molecular Biology lecture and lab, and cancer biology, both as an upper level elective and as a course for non-science majors. Previously, she developed and taught a class called Metabolism of Human Disease at Pine Manor College (Chestnut Hill, MA) as an adjunct instructor.

Dr. Mattaini spends some of her non-teaching time working extensively with the Tufts Full-time Lecturers Union as part of SEIU Local 509. Additionally, she has developed an expertise in alternative grading systems, and as a result, is a co-chair of the Organizing Committee for [the Grading Conference](#) and a founding member of the [Biology Grading for Growth](#) community of practice. For fun, she listens to lots of true crime podcasts and Agatha Christie audiobooks while crafting. She does all kinds of fiber crafts: knitting, crochet, cross stitch, and hand embroidery. If you visit her office, you will see many examples of her work! She also enjoys playing board games, tabletop RPGs, and video games with her husband, son, and friends.

She can be contacted at [Email: Katie Mattaini](#).

1: Bacterial Transformation

Summary

Bacterial transformation is the process by which bacteria take up free DNA from their surroundings. This process is commonly used to make bacteria take up and replicate recombinant DNA during molecular cloning.

Also known as

Transformation

Samples needed

Sample of DNA (typically recombinant DNA) and live bacterial cells

Method

Bacterial transformation is a molecular biology technique that utilizes the replication system of bacteria to replicate recombinant DNA. Bacterial transformation involves a 4 step process:

1. Generation of competent cells
2. Introduction and transformation of the DNA into the bacteria
3. Recovery
4. Plating

In step 1, competent cells are bacterial cells that are able to efficiently take up free DNA from their environment. Bacteria can be made competent through a variety of techniques such as incubation of the bacteria in a solution containing ions (such as calcium) that are thought to play a role in DNA adhering to the cell membrane and making it easier for DNA to enter during step 2.

In step 2, the DNA sample is added to the competent bacteria. The bacteria + DNA are then briefly exposed to a high temperature, a process known as heat shock. This allows for uptake of the DNA by the competent bacteria. Alternatively, a process known as electroporation may be used, where the bacteria are briefly subjected to a high-voltage electric field that creates pores in the cell membrane that allow for entry of the DNA.

In step 3, the bacteria are placed in liquid growth media and given time to recover from any cell membrane disruption that occurred during the heat shock or electroporation process. This also provides time for the bacteria to synthesize any enzymes encoded by the DNA, such as enzymes conferring antibiotic resistance.

In step 4, the bacteria are spread on plates of solid growth media and allowed to form colonies. Typically this growth media will contain antibiotics to help determine which bacteria have taken up the DNA. This antibiotic selection works by having an enzyme conferring antibiotic resistance encoded by the DNA sample so that only bacteria that took up the DNA will be able to survive and form colonies on the media containing antibiotics.

Controls

A no DNA control (water instead of DNA) is typically included as a negative control to ensure that no reagents are contaminated. Known plasmid DNA with the desired antibiotic resistance can serve as a positive control. Researchers will also sometimes include a comparison of colony growth between antibiotic and non-antibiotic containing media to verify that antibiotic selection occurred.

Interpretation

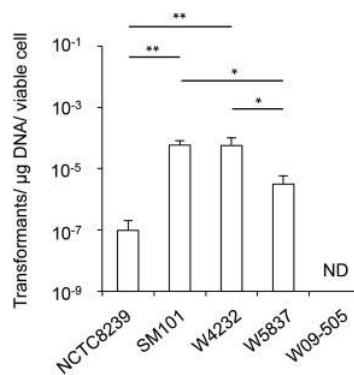


Figure 1. Transformation efficacy results for *C. perfringens* strains. Relevant section of caption for published figure reads “*C. perfringens* strains NCTC8239, SM101, W4232, W5837, and W09-505 were electroporated with the *Escherichia coli*-*C. perfringens* shuttle plasmid pXEH. The numbers of viable cells and transformants were determined by plating cells on a brain heart infusion (BHI) plate or a BHI plate containing 30 µg/ml erythromycin, respectively. All data represent the means ± SD from three independent experiments. ND indicates that transformants were below the detection threshold. *, $P < 0.05$ and **, $P < 0.01$ indicate significant differences between the two strains.” “Figure 1” by Yasugi et al.^[1] [Image description]

The authors sought to optimize bacterial transformation of *Clostridium perfringens*, a bacteria known to cause foodborne illness. In this experiment, the researchers first measured the ability to transform plasmid DNA into strains of *C. perfringens* known to cause foodborne illness (NCTC8239, W4232, W5837, and W09-505) compared to the better characterized SM101 strain. The researchers mixed plasmid DNA with competent *C. perfringens* cells, utilized the electroporation technique to transform plasmid DNA into cells, and determined the number of colonies on media containing the antibiotic erythromycin. The efficiency of the transformation was then calculated by dividing the number of colonies by the amount of DNA (in micrograms) and the amount of viable bacteria that were plated. The W4232 strain showed a similar transformation efficiency to the SM101 strain, while the NCTC8239 and W5837 strains showed significantly lower numbers of transformed bacteria compared to SM101. Notably, no transformed bacteria were observed for W09-505. Therefore, three out of the four strains tested showed decreased ability to uptake DNA compared to the SM101 strain.

Image Descriptions

Figure 1 image description:

A bar graph. The bar graph shows the number of transformant bacteria divided by micrograms of DNA divided by viable cells. SM101 and W4232 are both at 10^{-4} , while NCTC8239 is at 10^{-7} and W5837 is at 10^{-5} . W09-505 has no bar on the graph and instead says ND. ↩

Thumbnail

"*Vibrio cholerae* on TCBS agar.jpg" ↗ by Microrao is in the [Public Domain](#) ↗.

Image description: *Vibrio cholerae* on TCBS agar

Author

Rachel Ende, Tufts University

1. Yasugi, M, D. Motooka, S. Nakamura, and M. Miyake. 2020. Phosphorothioation of foreign DNA influences the transformation efficiency in *Clostridium perfringens* NCTC8239. *Anaerobe* 61:e102085. ↩

1: [Bacterial Transformation](#) is shared under a [CC BY-SA 4.0](#) license and was authored, remixed, and/or curated by LibreTexts.

2: Bradford Assay

Summary

This assay assesses the concentration of protein in a sample by measuring the absorbance shift from 465 nm (unbound dye) to 595 nm (dye bound to protein) using a spectrophotometer. As Coomassie Brilliant Blue G-250 dye binds to protein, the anionic binding results in a change from a brown/red color to a blue color which can be read by a plate reader to quantify the amount of sample at 595 nm after incubation. The more blue the more protein!

Also known as

Bradford Protein Assay, commercial names (i.e. Bio-Rad protein assay)

Similar techniques: BCA (Bicinchoninic acid) Assay

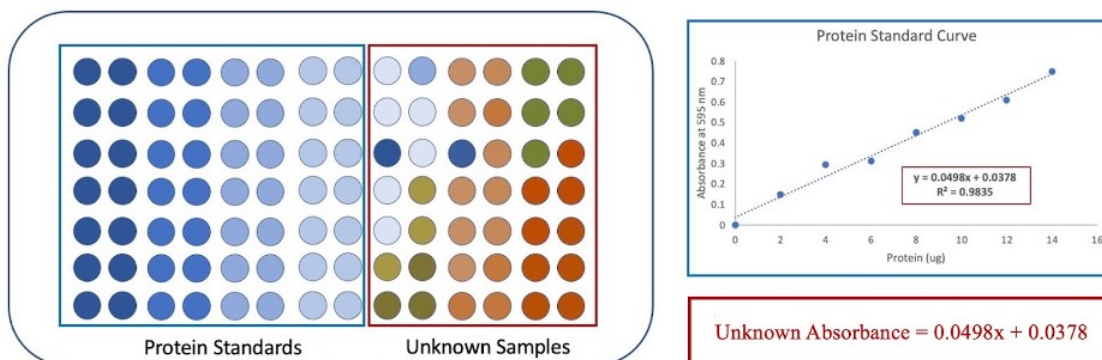
Samples needed

Samples for this assay can be any kind of solution where you want to measure protein content. Some common forms are cell lysate, purified protein, and plasma.

Method

The experimental protocol for this method is actually quite simple; protein samples are incubated with Coomassie Brilliant Blue dye for around 5-10 minutes in either a cuvette or a multiwell plate and then the absorbance is read by a spectrophotometer to assess protein binding.

To translate these readings into meaningful data you will first need to create a standard curve based on dilutions of your known protein concentrations which will be used to for comparison with your unknown sample absorbances for a final concentration measurement.



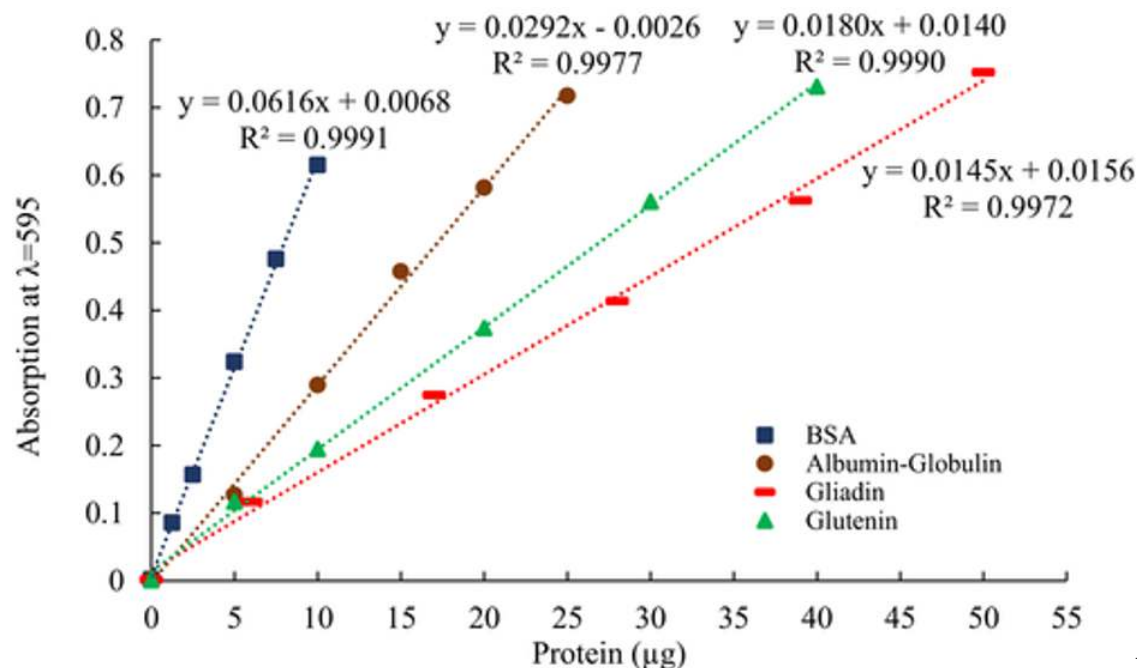
Figure

1. Hypothetical workflow, description in text. [\[Image description\]](#)

Controls

To properly run this assay you need two controls: a blank (a baseline solution with no protein) and a set of protein standards (samples with known protein concentrations much like a ladder in [gel electrophoresis](#)). The blank is used to establish a baseline for your sample buffer and the known protein dilutions are used to generate a standard curve to compare unknown absorbances.

Interpretation



Figure

2. Standard curve results of wheat protein fractions. Paper figure reads: “Linear graphs obtained with the Bradford assay using BSA (blue) and the purified wheat protein fractions Albumin–Globulin (brown), Gliadin (red) and Glutenin (green) as calibrants. Slope and y-intercept from the four derived equations. All values were measured in four replicates.” “Figure 1” of Rekowski et al.

^[1] [\[Image description\]](#)

These researchers used the absorbance of their known proteins (BSA, albumin-globulin, gliadin, glutenin), as control standards for their proteins of interest. BSA is the most commonly used protein standard but researchers may choose others, as shown above, if they are closer in composition to their unknowns of interest. The lines of best fit in these standards are then compared to assess protein concentration in their experimental groups.

Image Descriptions

Figure 1 image description:

The image on the left shows an example of a multiwell plate with a blue gradient covering the left half as the protein standard dilutions and a range of colors (blue, orange, brown, red) on the right side as experimental unknown samples. The right side shows an example of a standard curve with corresponding line of best fit. The y axis is absorbance at 595 nm and the x axis is amount of protein in ug. Below the standard curve, the equation of the line is used to assess the hypothetical unknown sample with the sample absorbance in place of the y value. ↩

Figure 2 image description:

This image shows multiple Bradford standard curves, ug of protein vs. absorbance at 595 nm, with corresponding equations. The lines with slopes from greatest to least are composed using standardized protein solutions of BSA (bovine serum albumin), albumin-globulin, gliadin, and glutenin. ↩

Author

Emilie Jones, Tufts University

1. Rekowski, A., Langenkämper, G., Dier, M., Wimmer, M. A., Scherf, K. A., & Zörb, C. (2021). Determination of soluble wheat protein fractions using the Bradford assay. *Cereal Chemistry*, 98(5), 1059–1065. ↩

2: [Bradford Assay](#) is shared under a [CC BY-SA 4.0](#) license and was authored, remixed, and/or curated by LibreTexts.

3: BrdU Assay

Summary

BrdU is a synthetic nucleoside analog, like the nucleoside thymidine, except the methyl group is replaced with a bromine atom. BrdU can be incorporated into DNA whenever thymidine normally would be, including during DNA replication and repair. Therefore, BrdU is often used to label cells that are undergoing or have recently undergone DNA replication.

Also known as:

Bromodeoxyuridine assay, 5-bromo-2'-deoxyuridine assay

Samples needed

Live cells are treated with BrdU, which incorporates into DNA being replicated. Subsequent steps depend on how sample will be visualized, for instance by [immunofluorescence microscopy](#) or [immunohistochemistry](#).

Method

Cells are treated with BrdU for a defined length of time. Any cells undergoing S-phase within the window when BrdU was present will have BrdU incorporated into their DNA. BrdU can be recognized by an anti-BrdU antibody, then visualized by the desired method. In order for BrdU to be accessible for the antibody to bind, the DNA has to be denatured.

Interpretation

If a cell is positive for BrdU, it was going through S phase when BrdU was present.

Special cases

To assess formation of single-strand DNA (ssDNA)

Normally, DNA has to be denatured for the BrdU antibody to detect any BrdU incorporated into the DNA. However, if a) BrdU labeling occurs over a long period so that all DNA is labeled and b) BrdU staining is performed under *non-denaturing* conditions, the only BrdU that will be accessible to the antibody will be in areas where DNA is single-stranded for some other reason (besides denaturing), like replication fork collapse. So, in this case, staining correlates with the amount of ssDNA in a sample.

Thumbnail

"BrdU staining (green).jpg" by Jason Snyder is licensed under [CC BY 2.0](#).

Description: BrdU (green) and arc (red) staining of neurons.

Author

Katherine Mattaini, Tufts University

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4: Cell Migration Assay

Summary

Cell migration assays are used to compare mobility of cells under different conditions. It tests a cell's ability to “crawl” across a solid substrate.

Also known as:

Scratch assay, wound-healing assay

Samples needed

A plate of confluent cells in a monolayer

Method

First, cells of interest are grown to confluence in a monolayer. Then, a pipette tip or another object is used to create a scratch through the monolayer, leaving an area of a consistent width open and free of cells. Finally, the “scratch” or “wound” is monitored and photographed to determine if one treatment group refills the gap faster than another.

Controls

The cell migration assay is nearly always used to compare mobility of cells +/- a treatment or intervention. Therefore, the usual mock treatment controls apply.

Interpretation

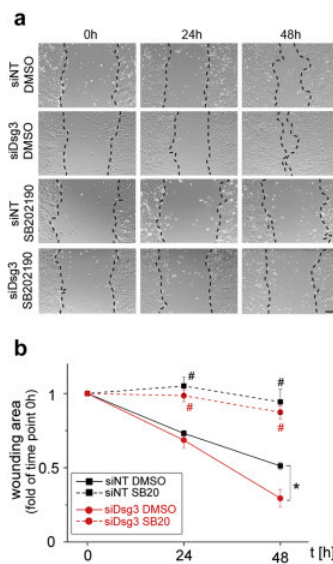


Figure 1. A cell migration assay in cells +/- siRNA for Dsg3 and +/- inhibitor of p38MAPK. Relevant section of caption for published figure reads: “(a) Representative bright-field images of HaCaT keratinocyte migration subjected to nontarget (siNT) or Dsg3 siRNA (siDSG3) and treated with solvent or with the p38MAPK inhibitor SB202190 (SB20). Bar is 150 μ m. (b) Wound closure expressed as the remaining area uncovered by the cells. The scratch area at time point 0 hours was set to 1 ($n = 4-6$; $*P < 0.05$, $\#P < 0.05$ vs. respective DMSO condition).” “Figure 3” by Rötzer et al.^[1] [Image description]

These microscopy images show that cells with decreased Dsg3 expression “heal the wound” faster by migrating to fill the scratched area. Therefore, the reader can conclude that one normal function of Dsg3 is to suppress cell migration. However, regardless of whether or not Dsg3 is expressed, if p38MAPK is inhibited, wound closure will not occur. Therefore, the migration-suppressing function of Dsg3 is dependent on p38MAPK activity.

Image Descriptions

Figure 1 image description:

Data from a cell migration assay.

Panel a: A grid of microscopy images of cells growing in culture. Columns show images from 0 h, 24 h, and 48 h after scratch was created. Rows from top to bottom are siNT + DMSO, siDsg3+DMSO, siNT+SB202190, siDg3+SB202190. The top two rows both show the scratch "closing," but the siDsg3 cells close the scratch faster and more completely. With SB202190, no closure occurs.

Panel b: A line graph with time in hours on the x axis and wounding area (fold of time point 0h) on the y axis. This graph quantifies what is seen from the microscopy images in panel a. The siDsg3+DMSO condition reaches wounding area of ~0.25 by 48 hours. The siNT+DMSO condition reaches ~0.5, and both of the SB202190 conditions are still very near 1. The difference between the two DMSO conditions is significant, as is the difference between each pair of samples with DMSO vs. SB202190 with the same siRNA.↵

Thumbnail

"Dh3-RD.jpg" ↗ by Dhifaf zeki is licensed under [CC BY-SA 4.0](#) ↗.

Description: This is image of Scratch wound healing assay of Rhabdomyosarcoma (RD) cell line (cancer cell line) under inverted phase contrast microscope.

Author

Katherine Mattaini, Tufts University

-
1. Rötzer, V., E. Hartlieb, J. Winkler, E. Walter, A. Schlipp, M. Sardy, V. Spindler, and J. Waschke. 2016. Desmoglein 3-dependent signaling regulates keratinocyte migration and wound healing. *Journal of Investigative Dermatology* 136:301-310. ↵
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5: Chromatin Immunoprecipitation

Summary

Chromatin immunoprecipitation is used to analyze protein-DNA binding interactions.

Also known as:

ChIP

Variants: ChIP-Seq, ChIP-qPCR

Samples needed

ChIP is used to determine whether a specific protein binds to DNA (either generally or at a sequence of interest), so the cells of interest are needed.

Method

Chromatin immunoprecipitation is used to analyze protein-DNA interactions in the cell. The first step in this method is to add a crosslinking agent (e.g., formaldehyde), which covalently binds proteins to DNA, preserving the natural protein-DNA interactions. The cells are lysed to release proteins, DNA, and protein-DNA complexes. Then, the chromatin is fragmented into smaller pieces, typically using sonication. After fragmentation, the lysates are incubated with beads bound to an antibody against the protein of interest. The antibody binds the protein of interest and, consequently, any DNA bound to it.

Next, the beads are washed to get rid of nonspecific binding, and the DNA is eluted from the beads. The crosslinks are reversed to isolate DNA from protein, and the DNA is purified. After purification of the DNA, there are several analysis methods that can be used. If the goal is to quantify the presence of a specific gene/region of DNA, [qPCR](#) can be used. (PCR can sometimes be used, but it is less common.) If the goal is to determine several possible genomic sequences present, sequencing methods can be used.

Controls

An input sample should always be used as a control, which is a sample that has not been incubated with the antibody against the protein of interest. The IP samples, which were incubated with the antibody against the protein of interest, are typically normalized to the input samples. Other controls can also be included. For example, an IgG control, where the sample is incubated with a nonspecific antibody, confirms the specificity of the protein-antibody interaction. Positive and negative locus controls use primers that amplify a locus where the protein of interest is either known to bind or known not to bind. These locus controls confirm that the experiment is working correctly and that the protein-DNA interaction is specific.

Interpretation

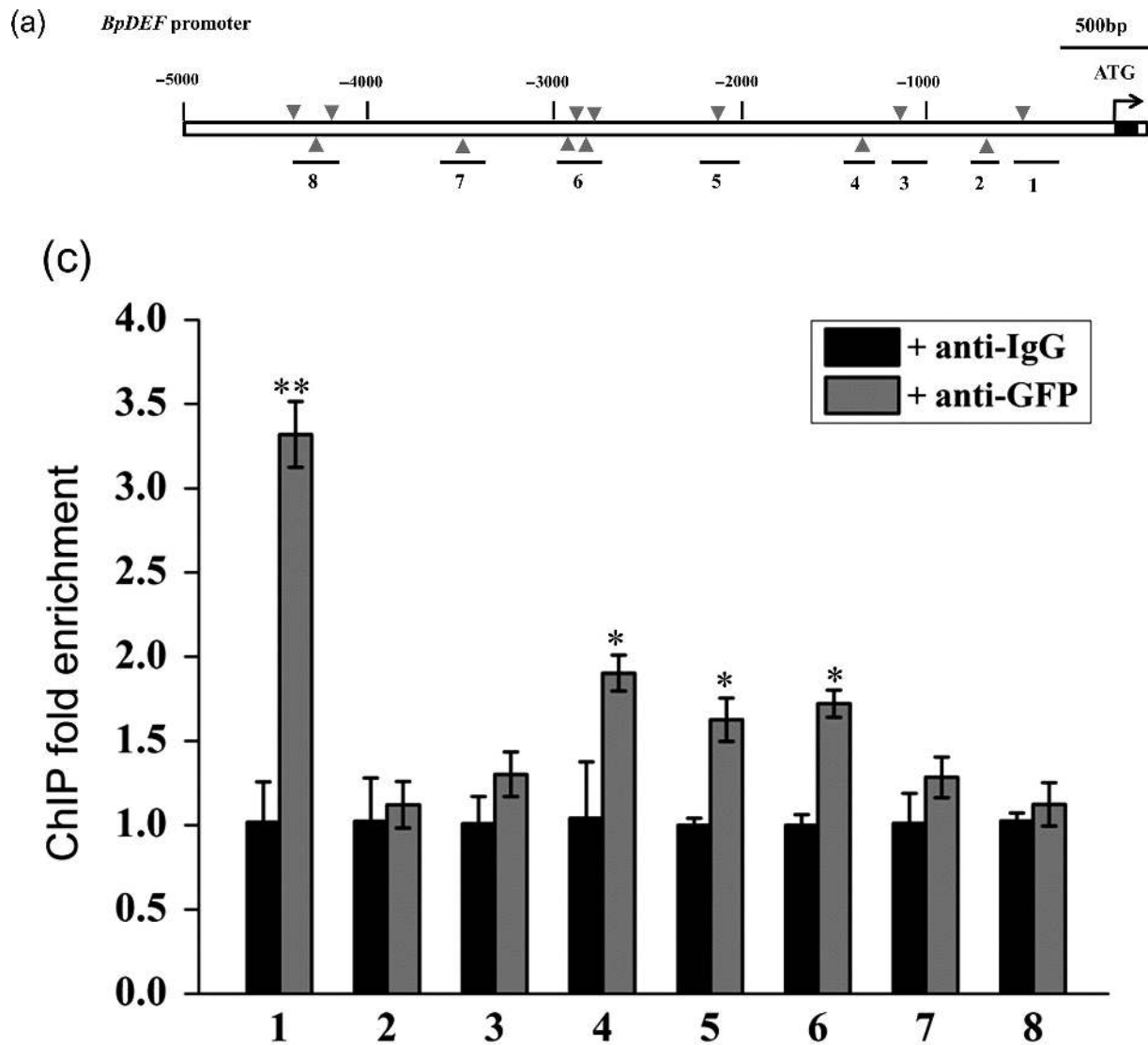


Figure 1. Chromatin immunoprecipitation enrichment of protein BpAP1 at the promoter of the *BpDEF* gene using qPCR. Relevant section of caption for published figure reads: “ (a) Promoter structure of *BpDEF*. Gray triangle represents GAR box motif, and DNA fragments near GAR box used for ChIP-PCR are shown. (c) ChIP fold enrichment of DNA fragments around the CAR boxes by ChIP-qPCR.” “Figure 7” by Wang al.^[1] [\[Image description\]](#)

BpAP1 is a protein in birch trees that can cause early flowering. In Wang et al., the authors wanted to determine the regulatory mechanism of the BpAP1 protein. To do so, they tested whether BpAP1 was enriched at the promoter of the *BpDEF* gene, which can promote flowering in birch trees. They tagged the BpAP1 protein with GFP and used an anti-GFP antibody to separate the BpAP1-DNA complexes.

Panel a shows a map of the *BpDEF* promoter, and each numbered black line represents a region the researchers tested for BpAP1 binding. At each numbered region, they used qPCR to quantify the amount of that sequence of DNA present in their IP samples. The gray bars in the graph depict the fold enrichment of BpAP1-GFP at each of the 8 regions of the *BpDEF* promoter. The black bars depict the IgG control. Note that the gray bars represent the IP samples over the Input samples for each of the 8 regions, and they are normalized to the IgG control. Based on the results from this experiment, the researchers can conclude that BpAP1 is enriched at (binds to) regions 1, 4, 5, and 6 of the *BpDEF* promoter.

Image Descriptions

Figure 1 image description:

Panel a: A map of the *BpDEF* gene promoter, which consists of 5000 base pairs upstream of the start codon. Gray triangles mark GArG box motifs, of which there are 13 total in the promoter. 8 numbered black lines (numbered from 3' to 5') mark regions that the researchers amplified using qPCR, and each region contains a GArG motif.

Panel b: A bar graph depicting ChIP fold enrichment in the anti-IgG and anti-GFP samples. The anti-GFP samples are normalized to the anti-IgG samples. The anti-GFP samples have the following enrichment of *BpAP1*-GFP at each region:

1: 3.3

2: 1.1

3: 1.2

4: 1.9

5: 1.6

6: 1.7

7: 1.3

8: 1.1

BpAP1 is significantly enriched at regions 1, 4, 5, and 6.↩

Thumbnail

["Chip sequencing2"](#) ↗ by [Chris Taplin](#) is licensed under [CC BY 3.0](#) ↗.

Description: A simple representation of small fragments of DNA bound by multiple different proteins, and one of those proteins bound by an antibody.

Author

Sara Crippen, Tufts University

-
1. Wang, S. H. Huang, R. Han, J. Chen, J. Jiang, H. Li, G. Liu, and S. Chen. 2019. BpAP1 directly regulates *BpDEF* to promote male inflorescence formation in *Betula platyphylla* x *B. pendula*. Tree Physiology 39: 1046-1060.↩
-

5: [Chromatin Immunoprecipitation](#) is shared under a [CC BY-SA](#) license and was authored, remixed, and/or curated by LibreTexts.

- 6: [Coimmunoprecipitation](#) by [Katherine Mattaini](#) is licensed [CC BY-SA 4.0](#).

6: Coimmunoprecipitation

Summary

Coimmunoprecipitation is used to determine if two proteins bind to one another in a sample.

Also known as:

Co-IP

Caution

Variant: Pull-down. Like a co-IP, a pull-down experiment determines if two proteins bind to one another, but unlike a co-IP, pull-downs do not involve an antibody-antigen interaction. Instead, the first protein of interest is bound to a column or precipitated ("pulled down") through some other affinity interaction, like 6x His tag with nickel or cobalt ions, or biotin with streptavidin.

However, in practice, the term "pull down" or "pull-down" is often used interchangeably with immunoprecipitation.

Samples needed

Usually co-IPs are used to determine if two proteins bind to one another in cells, so the cells of interest are needed.

Method

Coimmunoprecipitation allows a researcher to determine whether two proteins of interest (A and B) bind to one another in a cell lysate or other sample. First, an antibody that is specific to protein A is attached to beads that can be separated from a solution by centrifugation, magnet, or some other method. Then cells of interest are lysed to release the proteins. The lysate is incubated with the anti-A beads as well as control beads that have been attached to an antibody that does not bind protein A. During the incubation, the anti-A antibody binds protein A. Importantly, any other proteins that are also bound to protein A will also be bound to the beads through protein A. The exact buffer used for this incubation and the subsequent washes is important, because the buffer composition will determine what protein-protein interactions are maintained or broken.

Then the beads are washed to remove any proteins that are binding non-specifically, and finally the proteins still bound to the beads are eluted. This leaves the researcher with a solution of protein A and any other proteins that are bound to protein A. Since the question is whether protein A binds protein B, the sample is then subjected to [western blot](#) to detect protein B.

Controls

Researchers should always show whole lysate control, or a sample that has not been subjected to immunoprecipitation. This shows the reader how much of the protein of interest is present in the sample to begin with. Also, researchers should show a control where the pull-down is performed with an antibody that does not bind to the protein of interest.

Interpretation

IGROV-1 cells

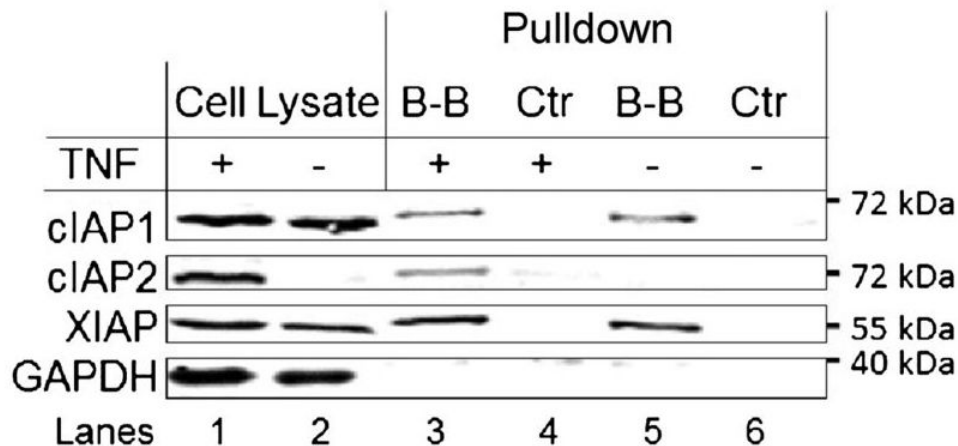


Figure 1. A Western blot showing the results of a pull-down experiment. Relevant section of caption for published figure reads: “C, cell lysates were prepared from IGROV-1... cells and incubated with 1 μ mol/L biotinylated-birinapant (B-B), or no compound (Ctr) for 16 hours. Magnetic streptavidin conjugated beads were then added and incubated for an additional 2 hours. Beads were collected by magnetic separation. Following washes, beads were boiled in SDS-PAGE sample loading buffer to elute bound proteins. Western blot analysis was performed to determine IAP association with birinapant.” “Figure 1” by Christopher A. Benetatos et al.^[1] [\[Image description\]](#)

Strictly speaking, one could argue that this is not an immunoprecipitation experiment, because there is no antibody involved. However, the protocol and reasoning are similar, and it is a form of pull-down assay. In this case, the researchers are asking whether the proteins cIAP1, cIAP2, and XIAP bind to the molecule birinapant. A molecule called biotin has been attached to the birinapant. Biotin binds very strongly to another molecule, avidin, which is attached to the magnetic beads. Therefore, the connections are: magnetic beads-avidin-biotin-birinapant-protein that binds birinapant.

Lanes 1 and 2 are the cell lysates before the pull-down is performed. This shows that cIAP1 and XIAP are expressed in the cells regardless of whether they are treated with TNF. cIAP2 is only detectable if the cells are treated with TNF. GAPDH serves as the loading control. Lanes 3 and 4 are the pull-down of lysates from cells treated with TNF; lanes 5 and 6 are the same but without TNF treatment. The control lysates have been treated with beads, but not with biotinylated birinapant. Therefore, as expected, they show no proteins eluted (lanes 4 and 6). Lane 3 shows three bands: one each for cIAP1, cIAP2, and XIAP. This indicates that in the cell lysates, those proteins bind the birinapant molecule. Lane 5 shows the same thing, but since the cells were not treated with TNF, there is no cIAP2 present.

Image Descriptions

Figure 1 image description:

A Western blot. Results are shown in table below.

Results of Western blot in Figure 1.

Sample	Cell lysate	Cell lysate	B-B pulldown	Ctrl pulldown	B-B pulldown	Ctrl pulldown
TNF treatment?	+	-	+	+	-	-
cIAP1	Band	Band	Band		Band	
cIAP2	Band		Band			
XIAP	Band	Band	Band		Band	
GAPDH	Band	Band				

Thumbnail

"Antibody IgG1 surface.png" [↗](#) by Tokenzero is licensed under [CC BY-SA 4.0](#) [↗](#).

Description: The structure of an antibody of subclass IgG1. Two light chains (green and pink) and two heavy chains (blue and yellow) are shown. Glycans between the heavy chains are shown in dark gray.

Author

Katherine Mattaini, Tufts University

-
1. Benetatos, C. A., Y. Mitsuuchi, J. M. Burns, E. M. Neiman, S. M. Condon, G. Yu, M. E. Seipel, G. S. Kapoor, M. G. LaPorte, S. R. Rippin, Y. Deng, M. S. Hendi, P. K. Tirunahari, Y. Lee, T. Haimowitz, M. D. Alexander, M. A. Graham, D. Weng, Y. Shi, M. A. McKinlay, and S. K. Chunduru. 2014. Birinapant (TL32711), a bivalent SMAC mimetic, targets TRAF2-associated cIAPs, abrogates TNF-induced NF- κ B activation, and is active in patient-derived xenograft models. *Molecular Cancer Therapeutics* 13:867-879. [↶](#)
-

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7: Column Chromatography

Summary

Column chromatography is a method for separating a mixture of substances based on their chemical and physical characteristics. In biological research, this is usually a mixture of proteins.

Also known as

Variants: Affinity chromatography, ion exchange chromatography, size exclusion chromatography

Samples needed

In biology, most column chromatography is performed on a solution containing a mixture of proteins

Method

Although column chromatography is used widely for many applications in chemistry, in biology and biochemistry, it is most often used to separate an aqueous mixture of proteins. The column is a tube filled with a stationary phase, also known as a resin. A solution called the mobile phase is then run through the column, either by gravity, or under pressure. The separation of the mixture into component proteins is based on the difference in how each protein interacts with the stationary phase vs. the mobile phase. For instance, a protein that interacts strongly with the stationary phase will take longer to move from the beginning to the end of the column, and subsequently, it will be eluted from the column later than another protein that interacts only weakly with the stationary phase. The liquid phase that comes off the end of the column can be collected in a series of fractions, which can then be used for further applications.

There are many variants of column chromatography that differ by the characteristic of the protein that is used to separate the mixture.

Ion exchange chromatography

Ion exchange chromatography separates proteins based on their overall charge. In this method, the stationary phase is either positively or negatively charged. The mobile phase is a solution that has a low salt concentration initially, but over the course of the run, the concentration of salt in the mobile phase is increased so that proteins that were initially strongly attracted to the stationary phase are eluted. For instance, if the stationary phase is negatively charged, proteins that have an overall negative charge will run through the column very quickly. However, proteins with an overall positive charge will interact with the stationary phase and therefore have a longer elution time. As the salt concentration increases, proteins that are weakly positively charged will elute first, followed by those that are strongly positively charged.

Size exclusion chromatography

As the name implies, size exclusion chromatography separates proteins based on their size and shape, and it is also called gel filtration. The resin in size exclusion chromatography is made of beads that have pores or channels running through them. The size of the beads and the channels can be used to fine-tune the separation achieved by this method. Large proteins will not be able to fit into the channels in the beads, and will therefore have the shortest elution times. However, smaller proteins will get caught up in the winding channels, and therefore, they will have a longer path through the column and longer elution times.

Caution

The outcome of size exclusion chromatography is the opposite of [gel electrophoresis](#), which can be confusing! In size exclusion chromatography, large proteins move the *fastest* through the column, whereas in gel electrophoresis, they move through the gel the *slowest*, since the gel acts as a simple sieve.

Affinity chromatography

Affinity chromatography achieves separation based on the affinity of one or a small number of proteins for a particular substance affixed to the stationary phase. For instance, if ATP is affixed to the stationary phase, ATP-binding proteins would interact with it,

and non-ATP-binding proteins would be quickly eluted. The ATP-binding proteins could be eluted by changing the mobile phase to a buffer containing “free” (not resin-bound) ATP. Another extremely common example is purification of proteins with a 6xHis epitope tag with a Ni²⁺ or Co²⁺ column. This method is known as immobilized metal affinity chromatography, or IMAC. Some of the N atoms in the histidine ring coordinate the metal ion, so that tagged proteins are bound to the column, and untagged proteins flow through. The tagged proteins can be eluted using a buffer containing imidazole, which has the same chemical structure as the ring in the amino acid histidine. Finally, if a column is derivatized with an antibody that binds a specific protein, that can also be considered affinity chromatography. However, when purifying a protein using an antibody, [immunoprecipitation](#) is often performed instead of chromatography.

Interpretation

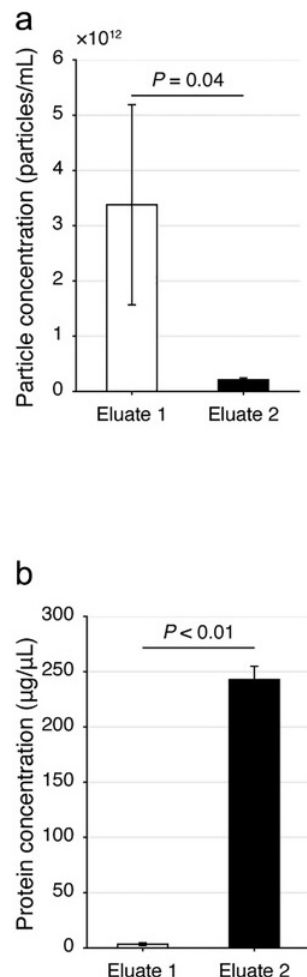


Figure 1. Analysis of extracellular vesicles (EVs) and proteins from human serum in eluate 1, collected early in a size exclusion chromatography experiment, vs. in eluate 2, collected later in the experiment. Relevant section of caption for published figure reads: “Evaluation of the dichotomic SEC method using human serum. (a, b) The particle (a) and protein (b) concentrations of the Eluate 1 and 2 with the dichotomic SEC method. Data are shown with mean $\pm$ SD, donor n = 3, Student’s t-test.” “Figure 1” by Gul et al.^[1] [\[Image description\]](#)

In this experiment, researchers showed that they successfully developed a relatively simple protocol to separate extracellular vesicles (EVs) from soluble proteins in human serum. Obviously, extracellular vesicles are far larger than individual proteins, so the use of size exclusion chromatography was a good choice. To make the protocol as straightforward as possible, all the mobile phase eluted from the column was divided into only two “fractions,” eluate 1 and eluate 2. Through extensive protocol optimization, the researchers were able to establish a cutoff between eluates 1 and 2 that resulted in good separation between the EVs and the proteins from the serum. In panel a, they measured the amount of particles, i.e. EVs, in each eluate using a special instrument. The instrument determined particle concentration through a mixture of light scattering and Brownian motion. In panel b, they measured protein concentration of each eluate using a Bradford assay. As expected, EVs were far more abundant in eluate 1,

since they are larger and would not get caught up in the channels of the resin. Eluate 2 contained most of the proteins not associated with the EVs, since they were much smaller.

Image Descriptions

Figure 1 image description:

Two column graphs. Graph a shows particle concentration. Eluate 1 contains $\sim 3.4 \times 10^{12} \pm 0.8 \times 10^{12}$ particles/ml and Eluate 2 contains $\sim 0.25 \times 10^{12} \pm 0.05 \times 10^{12}$ particles/ml. $p = 0.04$. Graph b shows protein concentration in $\mu\text{g}/\mu\text{l}$. Eluate 1 contains $\sim 5 \pm 1$ $\mu\text{g}/\mu\text{l}$ and Eluate 2 contains $\sim 240 \pm 20$ $\mu\text{g}/\mu\text{l}$. $p < 0.01$. ↩

Thumbnail

"Chromatography column.PNG" ↗ by Klaas1978 is in the [Public Domain](#) ↗.

Description: Chromatography column with sample in three different stages.

Author

Katherine Mattaini, Tufts University

1. Guo, J., C. Wu, X. Lin, J. Zhou, J. Zhang, W. Zheng, T. Wang, and Y. Cui. 2021. Establishment of a simplified dichotomic size-exclusion chromatography for isolating extracellular vesicles toward clinical applications. *Journal of Extracellular Vesicles* 10:e12145. ↩
-

7: [Column Chromatography](#) is shared under a [CC BY-SA 4.0](#) license and was authored, remixed, and/or curated by LibreTexts.

- [36: Western Blot](#) by [Katherine Mattaini](#) is licensed [CC BY-SA 4.0](#).

8: Cox Proportional Hazards Analysis

Summary

Cox proportional hazards analysis is a type of survival analysis. Generally speaking, a survival analysis is a mathematical method that models a) the length of time until an event occurs and b) whether that event occurs at all *as a function of* a number of variables of interest.

Disclaimer

I am going to write a **very** basic-level understanding here, since I am not a mathematician, nor are most of the target audience for this Encyclopedia. I found the videos on survival analysis from “[MarinStatsLectures-R Programming & Statistics](#)” on [Youtube](#) ↗ to be really useful. They’re what helped me write this entry rather than just having a vague idea of what was going on.

Also known as:

Cox PH, Cox regression

Samples needed

Dataset with independent variables of interest & time until target event

Method

As stated above, a **survival analysis** is a mathematical method that models a) whether an event occurs and b) the time it takes to occur *as a function of* a number of independent variables. So, for instance, you could use a dataset to predict how long a person is likely to survive after a lung cancer diagnosis (here, “event” = death) based on age at diagnosis, tumor stage at diagnosis, assigned sex, smoking history, etc.

Cox PH analysis is one particular type of survival analysis. The goal of Cox PH is to estimate something called a **hazard ratio (HR)** rather than to predict the length of survival. Let’s use the example of lung cancer above. A hazard ratio tells you *how many times more likely* a person from one group (i.e. a past or current smoker) is to die of lung cancer than a person another group (i.e. a never-smoker) at a given moment in time.

Caution

Not every analysis that uses this kind of math has death as an end point. When you’re looking at a Cox PH analysis, always ask yourself what the “event” or “outcome” is that’s being analyzed.

So, for the lung cancer example above, you could have a dataset something like this:

Table 1. Theoretical dataset

	Time from diagnosis to evaluation for event (months)	Did the event happen? (i.e. death)	Age at diagnosis	Tumor stage at diagnosis	(list all variables)
Patient 1	7	Yes → 1	68	IV → 4	
Patient 2	50	No → 0	75	II → 2	
... (list all patients)					
Dependent variables			Independent variables		

Step 1: Each patient would be a point & you would plot this data. (If you were doing just age vs. survival, it would be plotted in 2D space, if age *and* tumor stage vs. survival, it would be plotted in 3D space, etc.)

Step 2: Perform **regression analysis**. This basically means you use a computer program to figure out what constant values make the data points fit a certain equation. For example, you may be familiar with linear regression. The equation of a line is $y = mx + b$. So, if you plot a bunch of x-y points and use a computer program to find the best fit line, you can get the values of m (slope) & b (intercept). (See Figure 1.)

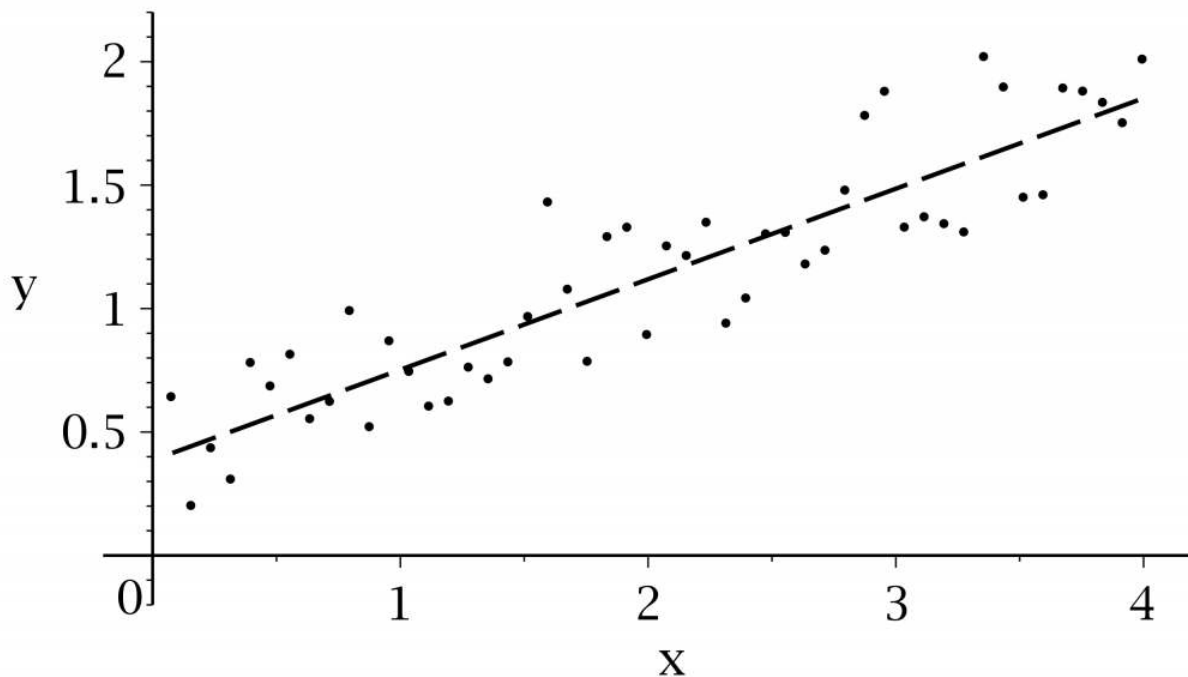


Figure 1. Example of linear regression analysis. “chow test illustration” by Kmhknh is licensed under CC BY 4.0.

Cox PH analysis is just like this, except you are fitting a much more complicated equation:

$$\text{Hazard}(t) = h_0(t) + e^{b_1x_1+b_2x_2+\dots} \quad (8.1)$$

In this equation, the x's are the independent variables like age at diagnosis, tumor stage at diagnosis, etc. The b's are the regression coefficients. They determine how important each independent variable is to the outcome.

So, what's the outcome? Your computer program gives you the b values from the equation above, which can be easily converted into a **hazard ratio** for each independent variable.

How do you interpret the HR? Say from the example above you found an HR of 1.2 when comparing smokers to non-smokers. That means that for people diagnosed with lung cancer, people who are current or previous smokers are 1.2 times more likely to die from their lung cancer at any given moment in time than those who have lung cancer but never smoked. (Or in other words, they're 20% more likely to die.) This is "adjusted for" their age at diagnosis, tumor stage at diagnosis, assigned sex, and any other independent variables that were included in the analysis. So, this HR tells you *just* the effect of their smoking status on survival.

Controls

Beyond the scope of this work!

Thumbnail

"Kaplan-Meier plot of AML survival.svg" ↗ by Michaelg2015 is licensed under [CC BY-SA 4.0](#) ↗.

Description: Kaplan-Meier plot of AML survival for aml data set from R survival package.

Author

Katherine Mattaini, Tufts University

Reviewed by Joshua Abston, Roger Williams University, B.S. Applied Mathematics

Content last updated on April 12, 2022

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9: CRISPR

Summary

CRISPR/Cas9 is a gene editing technique that allows scientists to “cut and paste” genes resulting in a gene “knockout” or insertion of new DNA or specific sequence changes, depending on how the technology is used.

Also known as:

CRISPR/Cas9, clustered regularly interspaced short palindromic repeats

Samples needed

Cells to be modified, guide RNA to lead Cas9 to DNA sequence of interest

Method

The CRISPR/Cas9 genome modification system was discovered within bacteria, which use CRISPR as a defense against previously encountered viruses. The system involves a guide RNA (gRNA) which is complementary to part of the gene of interest, and a nuclease, most commonly the Cas9 enzyme. Cas9 unzips the DNA and allows the RNA to bind to it, then Cas9 cuts both strands of DNA.

CRISPR/Cas9 can be used to achieve multiple different outcomes. When Cas9 creates a double-strand break, the cell will attempt to repair the sequence with normal cellular repair processes, namely homology-directed repair (HDR) or non-homologous end joining (NHEJ). When NHEJ is used, random small insertions and deletions occur, often resulting in a functional knock-out, i.e. the gene of interest no longer produces a functional product. This is the most common outcome if the researcher does not provide a plasmid containing a repair template. However, if the researcher provides a DNA sequence containing homology arms flanking the sequence of interest, the cell can use HDR to repair the break, resulting in a change to or insertion into the genome. This can result in a gene knock-in.

A plethora of CRISPR variants exist. One of these is base editing. In this application, either cytidine deaminase or adenosine deaminase is fused to a Cas9 nickase, resulting in either cytidine-to-uridine or adenosine-to-inosine changes. The ability of guide RNAs to target proteins to a particular location in the genome can also be used for applications other than genome editing. For instance, if a catalytically dead Cas9 is fused to a transcriptional activator or repressor, the CRISPR system can be used to control gene expression. GFP-dCas9 can be used to track the location of a particular DNA sequence.

Controls

The most important control should show scientists that the DNA has been modified in the correct place. This can involve DNA sequencing, or if the gene is being disrupted, use of a [western blot](#) to show loss of target protein expression.

Interpretation

The *TRAC* locus contains the gene for the constant region of T-cell receptor α (TCR α). In Eyquem et al., the authors set out to disrupt the *TRAC* locus and instead insert DNA encoding a chimeric antigen receptor (CAR), to create therapeutic CAR T cells for leukemia treatment. If the genomic modification was successful, the T cells should no longer express the T-cell receptor, and instead express the chimeric antigen receptor. Panel b shows the results of a [flow cytometry](#) experiment. When reading the flow results, each dot is one cell; the height on the y axis shows how much TCR is present on the cell's surface, whereas the distance along the x axis shows how much CAR is present. In the first graph, cells were supplied with the repair template containing the CAR gene, but no gRNA or Cas9. Essentially all cells show high levels of TCR and low CAR, as expected. In the second, cells were supplied with gRNA and Cas9, but no repair template. In this case, one would expect small insertions or deletions in the *TRAC* locus through the use of NHEJ. As expected, some cells in this group show much lower TCR levels, indicating that a functional knockout has been produced. In the final graph, all CRISPR components plus the repair template were supplied. In this case, there is a population of cells that show low TCR levels and high CAR levels, indicating that the locus has been successfully modified with the CAR DNA cassette.

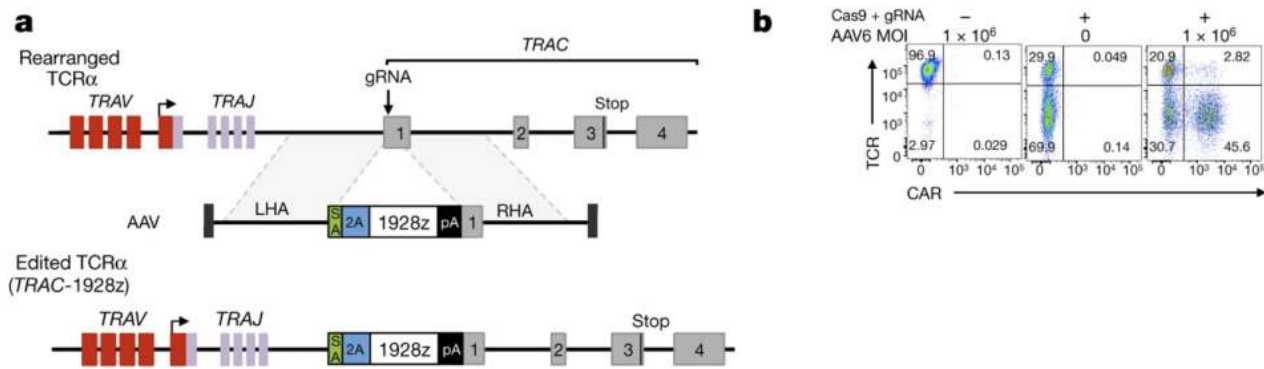


Figure 1. CRISPR modification of the *TRAC* locus in T-cells. Relevant section of caption for published figure reads: “a, CRISPR/Cas9-targeted CAR gene integration into the *TRAC* locus. Top, *TRAC* locus; middle, rAAV6 containing the CAR cassette flanked by homology arms; bottom, edited *TRAC* locus. b, Representative TCR/CAR flow plots 4 days after *TRAC* targeting.” “Figure 1” by Eyquem et al.^[1] [Image description]

Image Descriptions

Figure 1 image description:

Panel a: A schematic of a genomic locus. The top line is entitled “rearranged TCR α ,” with *TRAV* and *TRAJ* exons upstream of the *TRAC* locus. The gRNA targets exon 1 of the locus, while the STOP codon is found in exon 3. The second line is entitled “AAV” for the adeno-associated virus that contains the CAR gene cassette. It shows a left homology arm (LHA), the CAR cassette (SA-2A-1928z-pa-1), then right homology arm (RHA). The diagram shows that the LHA corresponds to the sequence upstream of exon 1 in the *TRAC* locus, and the RNA begins toward the 3’ end of exon 1.

Panel b: Density dot plots, the result of flow cytometry experiments. Percent of cells in each region shown in table below.

Summary of results in Figure 1, panel b.

			Cas9/gRNA - AAV MOI 1 $\times 10^6$	Cas9/gRNA + AAV MOI 0	Cas9/gRNA + AAV MOI 1 $\times 10^6$
Percent of cells	TCR high, CAR low (upper left)	96.9	29.9	20.9	
	TCR low, CAR low (lower left)	2.97	69.9	30.7	
	TCR low, CAR high (lower right)	0.029	0.14	45.6	
	TCR high, CAR high (upper right)	0.13	0.049	2.82	

↩

Thumbnail

"GRNA-Cas9.png" ↗ by marius walter is licensed under CC BY-SA 4.0 ↗.

Description: CRISPR Cas9 system

Authors

Original author: Anne E. Ebersold, Roger Williams University, April 25, 2022. Reviewed by Katherine Mattaini.

Later expanded & edited by Katherine Mattaini, Tufts University, August 21, 2024.

-
1. Eyquem, J., J. Mansilla-Soto, T. Giavridis, S. J. C. van der Stegen, M. Hamieh, K. M. Cunanan, A. Odak, M. Gönen, and M. Sadelain. 2017. Targeting a CAR to the TRAC locus with CRISPR/Cas9 enhances tumour rejection. *Nature* 543:113–117. [↩](#)

General sources:

[Medline Plus](#) ↗ [medlineplus.gov]

"How CRISPR works" ↗ [www.sciencenewsforstudents.org]

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10: DNA Microarray

Summary

A DNA microarray is a tool that can be used for multiple different kinds of experiments. This entry will summarize the use of DNA microarrays to compare the gene expression profiles of two different cell or tissue samples.

Also known as:

DNA chip, biochip, GeneChip

Note

The use of DNA microarrays to measure gene expression was very popular in the early 2000's. However, in the 2010's, RNA sequencing (RNA-seq) started to replace the microarray in large-scale gene expression experiments, since RNA-seq has several advantages over microarrays.^[1]

Samples needed

RNA from at least two cell or tissue samples

Method

First, [reverse transcription PCR](#) is used to reverse transcribe the RNA molecules present in each sample into cDNA. Then, the cDNA from sample A is labeled with one fluorescent molecule, and the cDNA from sample B is labeled with a second fluorescent molecule of a different color. The two samples are then applied at the same time to the microarray, which is a solid surface that contains many thousands of "spots" of DNA, each of a specific sequence. The cDNA from the samples is allowed to hybridize, or base-pair, with the DNA on the microarray. Then the microarray is "read" by measuring the intensity of each fluorescent molecule on each dot. Because the data often involves thousands of measurements, researchers often perform extensive analysis on it, including clustering analysis. When visualized, the data is often shown as a heatmap.

Controls

Many! Detailed description of controls for a microarray are beyond the scope of this work.

Interpretation

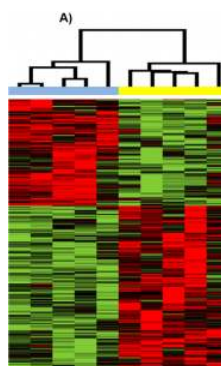


Figure 1. A heatmap showing the results of a gene expression experiment performed with a DNA microarray. The samples were early- and late-stage uterine serous carcinoma, a type of endometrial cancer. Relevant section of caption for published figure reads: "Hierarchical clustering of patient samples based on differentially expressed genes ($P < 0.01$) obtained from comparing late stage versus early stage in the USC group... In clustering dendrogram, blue stands for early stage samples while yellow stands for late stage samples. Red means up-regulated while green means down regulated." "Figure 1" by Mhawech-Fauceglia et al. ^[2] [\[Image description\]](#)

In Figure 1, each vertical column is a sample and each horizontal row is a gene. The heatmap shows only the genes that were significantly differently expressed across the ten samples, which the authors refer to as differentially expressed genes (DEGs). Then, the authors used a mathematical program to cluster, or group, the samples together based on which samples had the most

similar gene expression profiles. The clustering program assigned the samples to two distinct clusters based on their expression profiles. In one cluster are all the early-stage samples, and in the second cluster are all the late-stage samples. This shows that the early and late stage samples have distinct gene expression profiles.

Image Descriptions

Figure 1 image description:

A heatmap with 10 samples. The five early-stage samples on the left are in one cluster, and the other five, the late-stage samples, are in a second cluster. The early-stage cluster shows overexpressed genes in red at the top of the figure and green downregulated genes at the bottom of the figure. The genes that are overexpressed in the early-stage cluster are downregulated in the late-stage cluster and vice versa. ↩

Thumbnail

"DNA microarray.svg" ↗ by Guillaume Paumier is licensed under [CC BY-SA 3.0](#) ↗.

Description: A DNA microarray

Author

Katherine Mattaini, Tufts University

-
1. Wang, Z., M. Gerstein, and M. Snyder. 2009. RNA-Seq: a revolutionary tool for transcriptomics. *Nature Reviews Genetics* 10:57-63. ↩
 2. Mhawech-Fauceglia, P., D. Wang, J. Kesterson, K. Clark, L. Monhollen, K. Odunsi, S. Lele, and S. Liu. 2010. Microarray analysis reveals distinct gene expression profiles among different tumor histology, stage and disease outcomes in endometrial adenocarcinoma. *PLOS ONE* 5:e15415. ↩
-

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11: DNase Footprinting

Summary

A DNase footprinting assay is a technique used to determine the specific DNA sequence(s) to which a protein binds *in vitro*.

Also known as:

DNase I protection assay

DNA footprinting

Samples needed

To perform a DNase footprinting assay, you need:

1. A protein sample, which can be either purified protein or a mixture isolated from cells or tissue.
2. A DNA probe labeled with either a fluorescent or radioactive tag.
3. DNase I or another enzyme that will cleave phosphodiester bonds in the DNA backbone.

Method

In this method, a DNA fragment is radioactively or fluorescently labeled at one end and incubated with the protein of interest. The mixture is then treated with DNase I, an enzyme that randomly cuts the backbone of DNA. The region where the protein is bound is protected from cleavage, creating a "footprint." When the resulting DNA fragments are separated by size using gel electrophoresis, the protected region appears as a gap or missing band in the pattern, revealing the exact binding site of the protein on the DNA. To determine the exact DNA sequence that is protected, [Sanger sequencing](#) reactions can be electrophoresed next to the footprinting reactions.

Controls

Typically, a control with DNA + DNase I but no protein is included, because DNase I won't cleave equally at all DNA sequences. This reaction is compared to DNase I reactions that include protein.

Interpretation

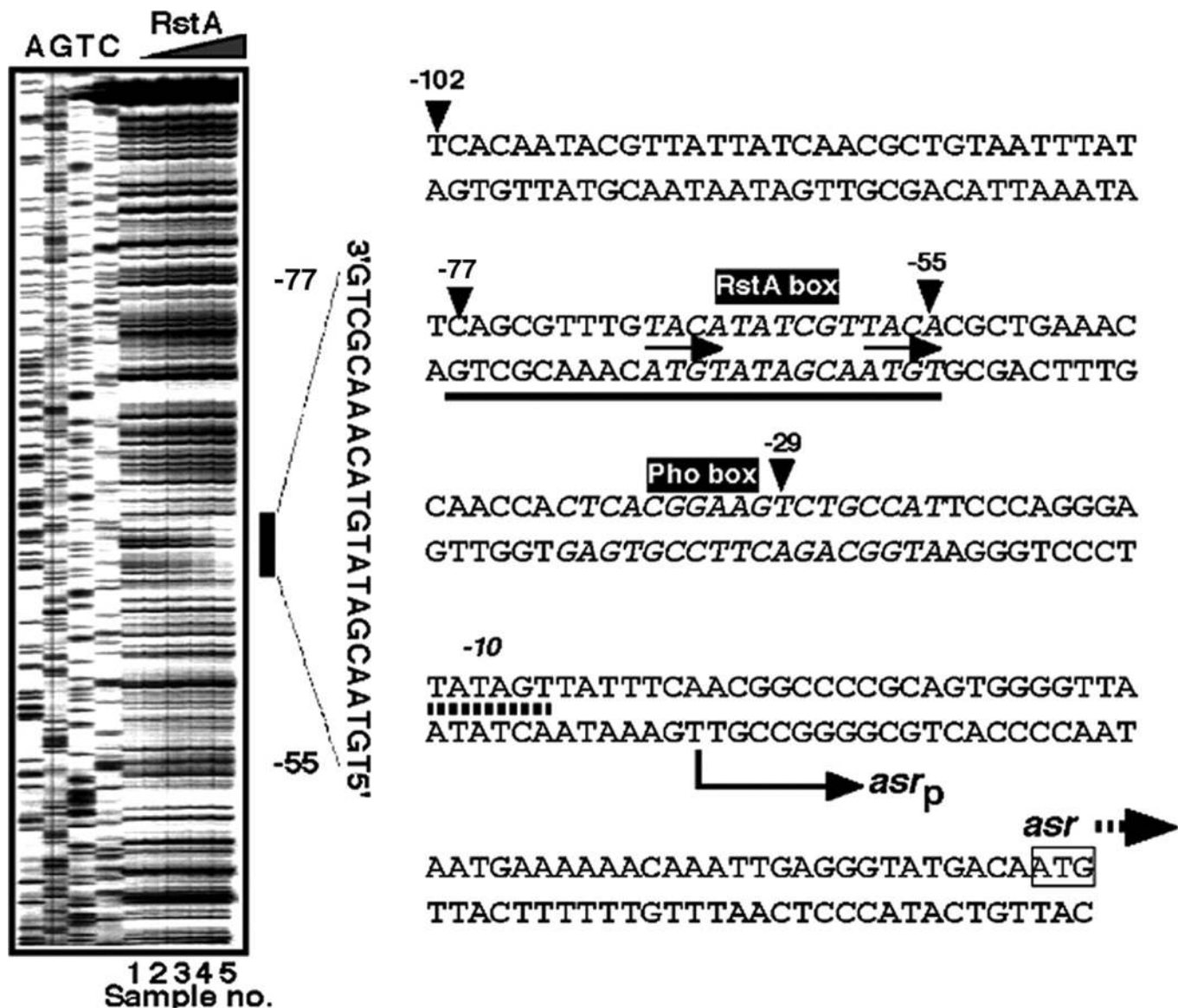


Figure 1. DNase I footprinting assay of the *asr* promoter. A fluorescently-labeled DNA probe of the *asr* promoter fragment was incubated with increasing concentrations of purified RstA (lane 1, 0 pmol; lane 2, 10 pmol; lane 3, 20 pmol; lane 4, 40 pmol; lane 5, 80 pmol) and subjected to DNase I footprinting assays. Lanes A, T, G, and C represent the respective DNA fragments obtained from Sanger sequencing. The DNA sequence to the right (-77 to -55) is referenced relative to the transcription initiation site. “Figure 3” by Ogasawara *et al.* ^[1] [\[Image description\]](#)

This figure shows that as the concentration of the RetA protein increases, the DNA sequence from -77 to -55 is protected from cleavage by DNase I. The four leftmost lanes show the results of [Sanger sequencing](#) reactions with ddATP, ddGTP, ddTTP, and ddCTP included. The five rightmost lanes show the results of DNase I digestion reactions, with a protected site visible at the two highest concentrations of RetA. The indicated sequence (-77 to -55) is protected from DNase I digestion when bound to the RstA protein, resulting in a lack of bands at the sizes that would correspond to these fragments.

[Image Descriptions](#)

Figure 1 image description:

A DNase I footprinting assay. There are nine gel lanes. The first four lanes are Sanger sequencing reactions that can be used to determine the sequence of the *asr* promoter DNA. The last resulting of Sanger sequencing reactions with ddATP, ddGTP, ddTTP, and ddCTP included. The bands correspond to the DNA sequence of the *asr* promoter fragment. The last five lanes show the results of DNA fragmentation that is carried out in the presence of DNase I. There is a footprint at the sequence that is bound by the RstA protein (at positions -77 to -55). ↩

Thumbnail

"Binding of SmeT to DNA" ↗ by Hernández et al. is licensed under [CC BY-SA 4.0](#) ↗.

Author

Mitch McVey, Tufts University

1. Ogasawara, H., A. Hasegawa, E. Kanda, T. Miki, K. Yamamoto, and A. Ishihama. 2007. Genomic SELEX search for target promoters under the control of the PhoQP-RstBA single relay cascade. *Journal of Bacteriology* 189:4791-4799. ↩

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12: Electrophoretic Mobility Shift Assay

Summary

An Electrophoretic Mobility Shift Assay is a technique used to study protein-nucleic acid interactions *in vitro*.

Also known as:

EMSA

Gel shift

Samples needed

To perform an EMSA, you need:

1. A protein sample, which can be either purified protein or a mixture isolated from cells or tissue.
2. A DNA probe labeled with either a fluorescent or radioactive tag. An EMSA can also be performed with an RNA probe if you're interested in RNA-protein interactions.

Method

To perform an EMSA, a short DNA fragment is labeled with a fluorescent or radioactive tag to make it detectable. This labeled DNA is then mixed with a protein extract or purified proteins, allowing the proteins to bind to the DNA. The mixture is loaded onto a non-denaturing agarose or polyacrylamide gel and subjected to [electrophoresis](#). During this process, the DNA-protein complexes migrate more slowly through the gel compared to free DNA fragments, resulting in a "shift" in the position of the bands. After electrophoresis, the gel is visualized using autoradiography or fluorescence to detect the shifted bands, which indicate the presence of DNA-protein complexes.

Controls

Validation of the binding reaction can be carried out in several ways. First, an antibody to the protein being tested can be included in one of the binding reactions. The binding of the antibody to the protein-DNA complex results in a larger complex that migrates even more slowly than the protein-DNA complex (this is often referred to as a 'supershift.' Second, non-labeled competitor DNA can be included in the binding reaction in molar excess over the labeled probe DNA. The competitor DNA will outcompete the binding of the protein to the labeled DNA, resulting in a less intense protein-DNA band shifted band.

Interpretation

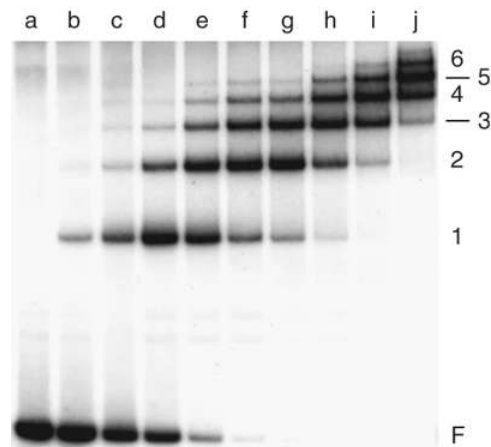


Figure 1. Multiple binding events of the *Escherichia coli* lactose repressor with a 203-bp *lac* promoter DNA. Relevant section of caption for published figure reads: "All samples contained 1.91×10^{-9} M DNA; samples b–j contained 0.08, 0.16, 0.32, 0.48, 0.61, 0.83, 0.99, $1.32, 1.66 \times 10^{-8}$ M repressor protein, respectively. The binding buffer contained 10 mM Tris (pH 8 at 20 °C), 1 mM EDTA, 50 mM KCl, 100 $\mu\text{g ml}^{-1}$ BSA, 5% vol/vol glycerol. Samples were resolved on a 5% wt/vol polyacrylamide gel cast and run at room temperature ($20 \text{ }^{\circ}\text{C} \pm 2 \text{ }^{\circ}\text{C}$) in 45 mM Tris–borate (pH 7.8), 2.5 mM EDTA. Electrophoretic species are designated F, free DNA or numbered (1–6) to indicate the repressor: DNA ratio of the corresponding complex. Under these conditions, repressor binds nonspecific sites as well as its specific operator sequences." "Figure 3" by Hellman and Fried.^[1] [\[Image description\]](#)

This figure shows that as the concentration of the *lac* repressor protein in a binding reaction increases, more proteins bind to the *lac* promoter sequence on the DNA probe. This results in increasing numbers of higher molecular weight protein-nucleic acid complexes that migrate more slowly during gel electrophoresis. The intensity of the band corresponding to the free probe (not bound to any protein) decreases with increasing protein concentration (moving left to right on the gel). With extremely high protein concentrations, the protein starts to bind to DNA sequences that are not the *lac* promoter, even though these protein-DNA interactions have lower affinity.

Image Descriptions

Figure 1 image description:

An EMSA assay. There are ten gel lanes, showing protein-DNA complexes that form when the *lac* operon promoter DNA is incubated with increasing concentrations of the *lac* operon repressor. Larger complexes migrate slower through the gel than smaller complexes.

Results of EMSA

Lane	Concentration of repressor protein (X10 ⁻⁸ M)	Intensity of free probe band	Repressor : DNA ratio of bound probe complexes present	Repressor : DNA ratio of bound probe in main complex present
a	0	Very strong	0	0
b	0.08	Strong	1,2,3	1
c	0.16	Medium	1,2,3,4,5	1
d	0.32	Medium	1,2,3,4,5	1
e	0.45	Weak	1,2,3,4,5	1
f	0.61	Very weak	1,2,3,4,5	2
g	0.83	None	1,2,3,4,5	2
h	0.99	None	1,2,3,4,5,6	3
i	1.32	None	2,3,4,5,6	4
j	1.66	None	3,4,5,6,7	5

↩

Thumbnail

"EMSA schematic" ↗ by [Linsalrob](#) ↗ is licensed under [CC BY-SA 4.0](#) ↗.

Author

Mitch McVey, Tufts University

1. Hellman, L. M. and M. G. Fried. 2007. Electrophoretic mobility shift assay (EMSA) for detecting protein-nucleic acid interactions. Nature Protocols 2:1849-1861. ↩

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13: Flow Cytometry

Summary

Flow cytometry is a biotechnology that can be used for many applications. This entry focuses on its use to detect the level of biomarkers present on individual cells in a sample.

Also known as:

Fluorescence-activated cell sorting, FACS

Caution

Technically speaking, FACS is a derivative of flow cytometry that physically separates cells into different populations based on their characteristics. By flow cytometry it's also possible to simply observe those different characteristics without separating the cells.

Samples needed

Single-cell suspension.

Cells will be treated in different ways depending on the measurement being made. Often, cells are [immunostained](#) for cell-surface biomarkers (i.e. proteins). Flow cytometry can be used with immunostaining for intracellular biomarkers if they are first fixed and then permeabilized to allow the antibody to enter the cells.

Method

Here, we are describing the use of flow cytometry to detect cell-surface proteins. The cells are first separated to form a single-cell suspension in an appropriate buffer. Then they are immunostained for one or several cell-surface markers. The number of cell surface markers that can be detected in one experiment is limited only by the number of different wavelengths of light that can be simultaneously detected and resolved by the particular flow cytometer being used.

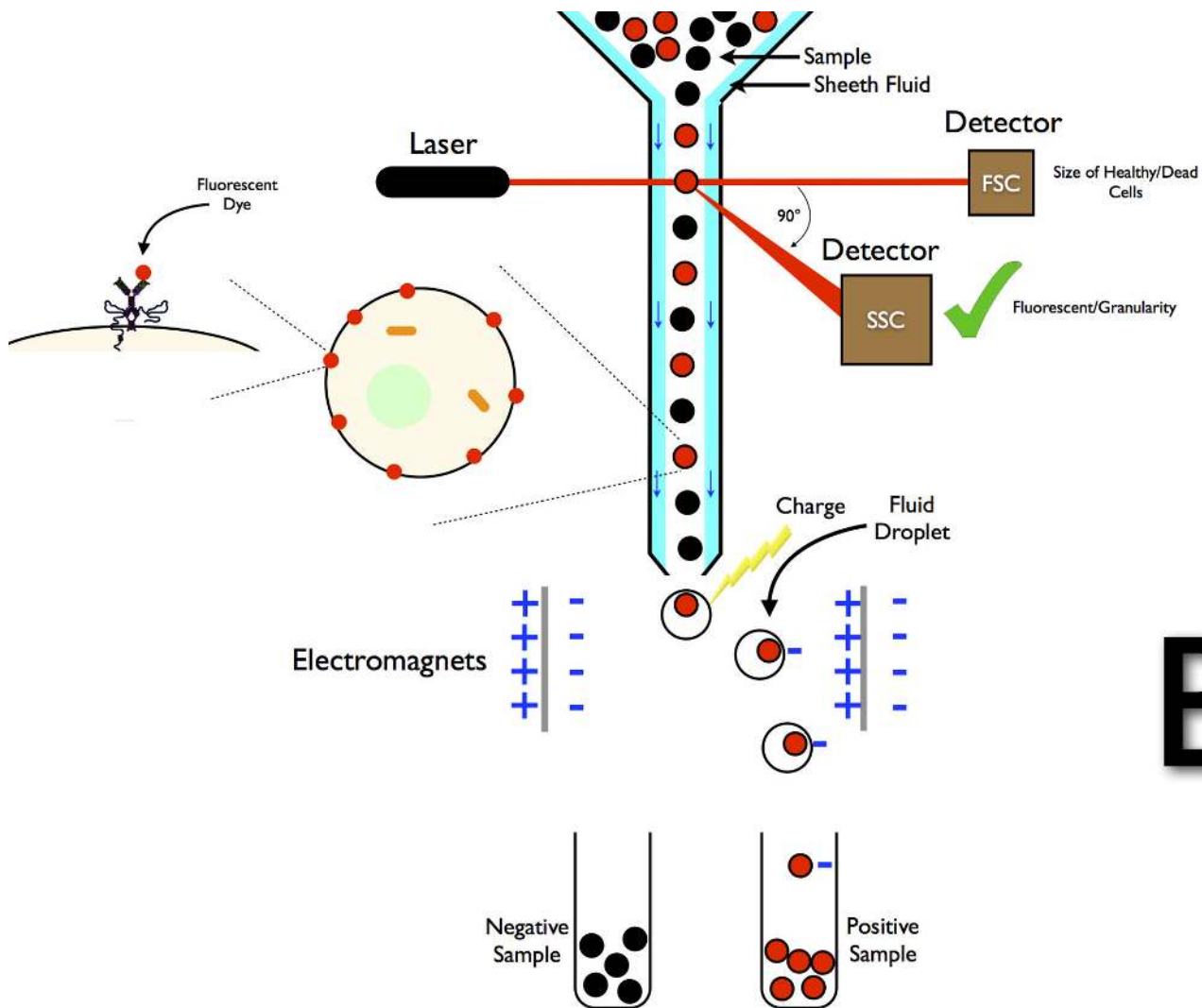


Figure 1. Simplified schematic of flow cytometer being used for fluorescence-activated cell sorting (FACS). The cells in this sample have been immunostained for a cell-surface protein. This process uses an antibody that binds the cell-surface protein of interest covalently attached to a fluorescent dye. Therefore, in the image, the cells with the protein of interest are shown in red. “Cell Sorting Using Flow Cytometry and Droplet Technology” by Sari Sabban is licensed under CC BY-SA 3.0. [Image description]

The cells are then allowed to pass through the flow cytometer one at a time. For each cell, two parameters called forward scatter (FSC) and side scatter (SSC) are measured, which give information about cell size and internal complexity (i.e. “granularity”), respectively. The intensity of each fluorescence signal is also measured to determine how much of each biomarker is present on the cell surface.

Controls

Recommended controls are numerous and beyond the scope of this Encyclopedia. However, they are rarely shown in published studies unless the study specifically aims to develop a new method.

Interpretation

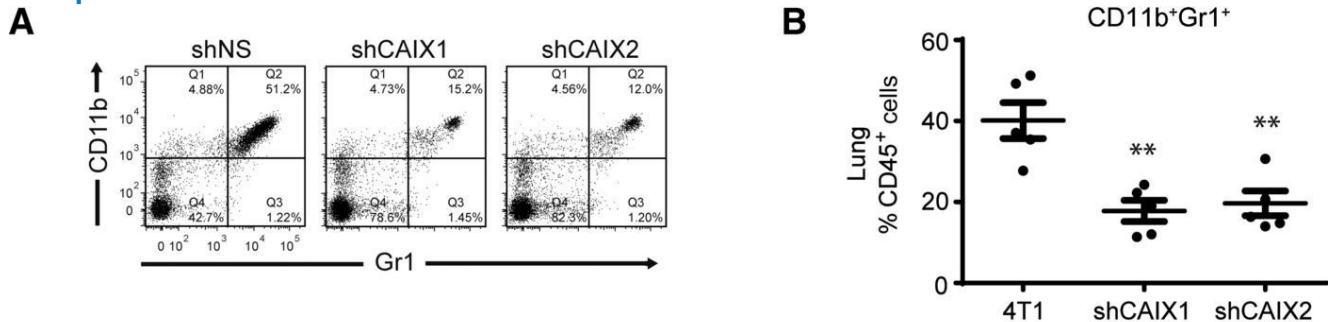


Figure 2. Results of a flow cytometry experiment. Relevant section of caption for published figure reads: “A, representative flow cytometry plots depicting tissue CD11b⁺Gr1⁺ recruitment 11 days after orthotopic inoculation of 4T1, 4T1 shCAIX1, and 4T1 shCAIX2 cells. B, quantification of CD11b⁺Gr1⁺ recruitment to the lungs (top) and spleen (bottom) of individual mice for each group as a proportion of the infiltrating CD45⁺ leukocyte population, $n = 5$ mice per group.” “Figure 3. CAIX is required for G-CSF-mediated granulocyte recruitment to the lungs of tumor-bearing mice.” by Shawn C. Chafe et al.^[1] [Image description]

In this experiment, the study authors have established orthotopic tumors in mice using 4T1 mouse breast cancer cells. The 4T1 cells express either a control shRNA (shNS), or one of two shRNAs against CAIX. The authors want to know how the level of CAIX expression in the tumor affects the presence in the mouse lungs of a certain subset of cells that highly express the proteins CD11b and Gr1 on their surface. To do this, the authors removed the lungs from the mice and processed them to a single-cell suspension. The cells were then immunostained for CD45, CD11b, and Gr1. CD45 is a general leukocyte marker, and Panels A and B are only showing cells from the lung that have high CD45 staining; CD45 low cells are excluded from the analysis. In the scatterplots in Panel A, each point on the graph is a cell, positioned based on the intensity of the CD11b and Gr1 staining. The researchers have divided the plot into quadrants that show the cells as “low” or “high” for each of the two biomarkers. Panel B is a dot plot that quantifies the results from Panel A by showing the overall % of the CD45⁺ cells in each sample that are CD11b⁺Gr1⁺. Based on this data, the authors conclude that when the primary tumor does not express CAIX, fewer CD11b⁺Gr1⁺ cells are found in the lung.

Image Descriptions

Figure 1 image description:

Single cells passing through a funnel-shaped structure in a flow cytometer. Cells are either red to show the presence of the cell surface protein or black to show its absence. As the cells pass through the narrow part of the “funnel,” they are hit by a laser, and scattered light is detected by one of two detectors, one for forward scatter and one for side scatter. As cells come out of the “funnel” in droplets, the droplets are charged, and can be electromagnetically attracted into one of two tubes: one containing only red cells and one containing only black cells. Side panel shows a cartoon of an antibody attached to a red fluorescent dye binding to a cell-surface protein on a cell. ↩

Figure 2 image description:

Panel A shows three scatterplots. The % of cells (points) in each quadrant of the scatterplot are shown in the table below.

Summary of results in Figure 2, panel a.

		shNS	shCAIX1	shCAIX2
Percent of cells	CD11b high, Gr1 low (upper left)	4.88	4.73	4.56
	CD11b high, Gr1 high (upper right)	51.2	15.2	12.0
	CD11b low, Gr1 high (lower right)	1.22	1.45	1.20
	CD11b low, Gr1 low (lower left)	42.7	78.6	82.3

Panel B shows a dot plot comparing the % of CD45 positive cells that are CD11b⁺Gr1⁺ in the three samples. It is a quantification of the scatterplots in Panel A. The 4T1 sample shows an average of 40%, whereas both of the shCAIX samples show averages around 20%. ↩

Thumbnail

"Flow cytometry histogram.png" ↗ by Kwz at Polish Wikipedia is licensed under the [GNU Free Documentation License](#) ↗, Version 1.2 or any later version published by the [Free Software Foundation](#) ↗; with no Invariant Sections, no Front-Cover Texts, and no Back-Cover Texts.

Description: Histogram of a blood sample studied with a flow cytometer.

Author

Katherine Mattaini, Tufts University

-
1. Chafe, S. C., Y. Lou, J. Sceneay, M. Vallejo, M. J. Hamilton, P. C. McDonald, K. L. Bennewith, A. Möller, and S. Dedhar. 2015. Carbonic anhydrase IX promotes myeloid-derived suppressor cell mobilization and establishment of a metastatic niche by stimulating G-CSF production. *Cancer Research* 75:996-1008. ↩
-

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14: Gel Electrophoresis

Summary

Gel electrophoresis is a method that separates biological macromolecules based on their size and sometimes (if a non-denaturing gel is used) their 3D structure.

Also known as

Variants: DNA/RNA gel, protein gel, agarose gel, polyacrylamide gel, SDS-PAGE

Samples needed

Mixtures or pure samples of either nucleic acids or proteins

Method

To separate a mixture of biological macromolecules, gel electrophoresis takes advantage of the sieving effects of a polymer gel. Generally, an electric field is applied so that negatively charged molecules travel toward the bottom of the gel. Smaller molecules move more quickly, as they pass through the crosslinked polymer more readily than large molecules, so small molecules end up near the bottom of the gel. (Note that nucleic acids are inherently negatively charged due to the phosphate backbone, but proteins need an appropriate buffer to be coated in negative charge.)

Gel electrophoresis is most frequently used as an analytical method, i.e. to determine the size and number of molecules in a sample. However, sometimes specific bands are cut out of the gel and purified for further use.

Gel electrophoresis can be used to separate samples containing nucleic acids and/or proteins. When viewing an image of a gel, it is important to determine which type of molecules are being visualized. After being run, gels are normally clear and colorless. Therefore, further steps are necessary to visualize the bands in the gel. For nucleic acids, a common method is the use of intercalating agents like ethidium bromide, which is visualized under a UV light. For proteins, a common method is using Coomassie brilliant blue dye, which binds to proteins relatively non-specifically.

The two most common types of gels used in electrophoresis are made of either agarose or polyacrylamide. Agarose is mainly used for separating nucleic acids with relatively large size differences of at least a few 100 nucleotides. Polyacrylamide gives greater resolution and is normally used for proteins and for separating nucleic acids with much smaller size differences. For instance, polyacrylamide gels are used for sequencing gels, where nucleic acids that differ by only one nucleotide need to be separated. Both types of gels can be made using different percentages of the polymer to achieve different separation characteristics.

Gel electrophoresis can be run under denaturing or non-denaturing (native) conditions, depending on whether the researcher wants to maintain secondary and higher structures of the macromolecules and interactions between macromolecules. For instance, SDS-PAGE is a very common technique used to separate denatured proteins, so that the proteins run through the gel based on only their molecular weight and not their structures. SDS-PAGE stands for sodium dodecyl sulfate polyacrylamide gel electrophoresis. SDS is an ionic detergent, which along with a reducing agent like Dithiothreitol (DTT) or β -mercaptoethanol (BME), and a heating step, ensures that proteins are fully denatured before running them on the gel. SDS also coats proteins in negative charge proportional to their molecular weight, so that they move toward the positive electrode at the bottom of the gel.

When imaging nucleic acids on a gel, supercoiling also has to be taken into account. Most commercially sold molecular weight ladders for nucleic acids consist of linear molecules, so a piece of linear nucleic acid will run “true to size” in comparison with the ladder. However, a nicked, circular, double-stranded nucleic acid will run slower than expected. Conversely, a supercoiled, circular, double-stranded nucleic acid will run more quickly than expected. This is true regardless of whether the supercoiling is positive or negative.

Gel electrophoresis is often used as an early step in other methods that detect one specific macromolecule, like [Southern blot](#) (DNA), [northern blot](#) (RNA), and [western blot](#) (protein).

Controls

Molecular weight markers made of macromolecules of known size should be run alongside of samples to relate distance traveled in the gel to molecular weight of bands.

Interpretation

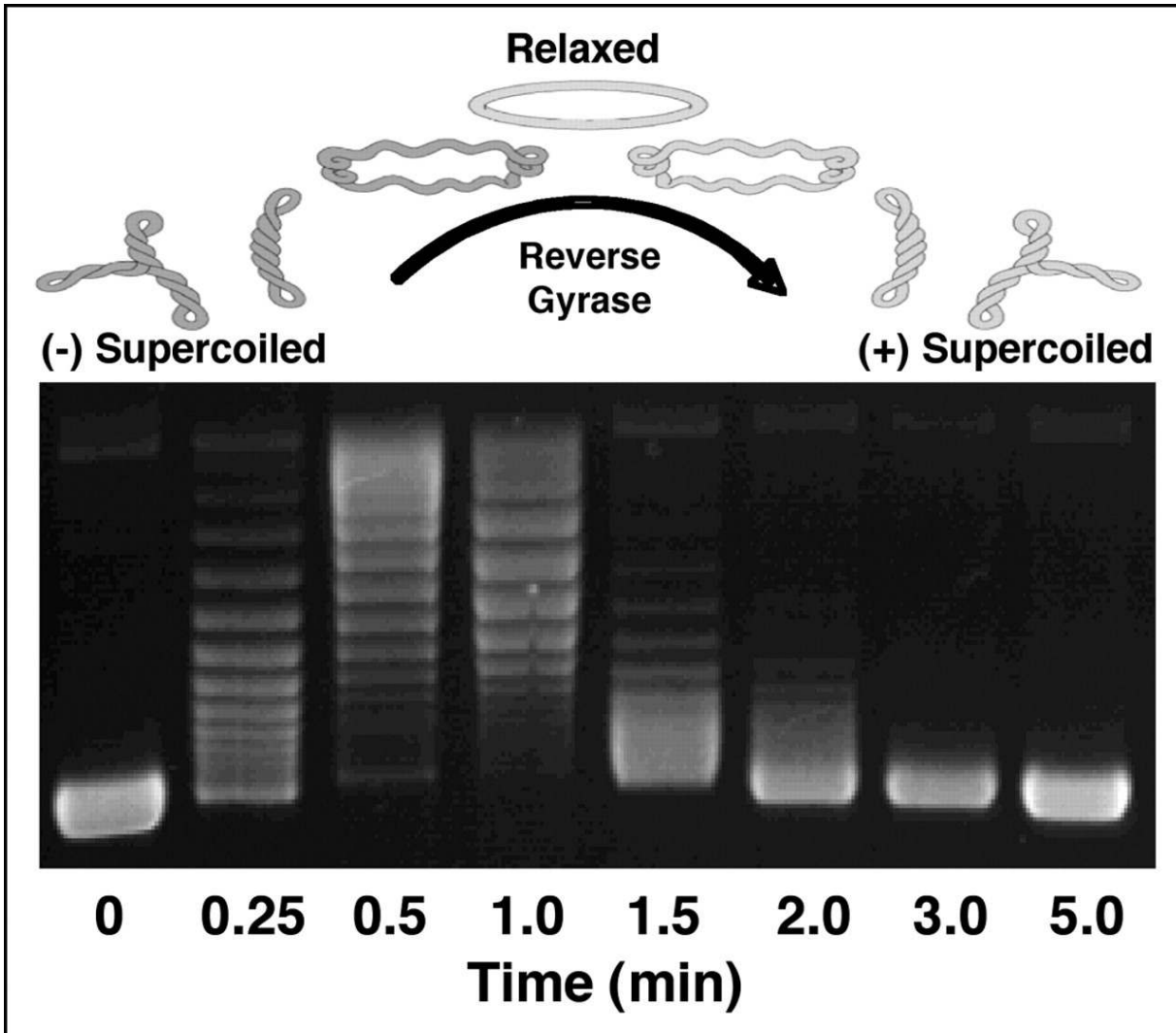


Figure 1. Ethidium bromide stained agarose gel loaded with plasmid (circular dsDNA). Relevant section of caption for published figure reads: “Figure 2. Generation of positively supercoiled DNA by *A. fulgidus* reverse gyrase. The positions of negatively (-) supercoiled, relaxed, and positively (+) supercoiled DNA are indicated. Negatively supercoiled pBR322 plasmid DNA was incubated with reverse gyrase for the indicated times. The extent of positive supercoiling was monitored by agarose gel electrophoresis.” “Figure 2” by McClendon et al.^[1] [\[Image description\]](#)

In this experiment, researchers purified a circular, dsDNA molecule (plasmid) called pBR322 from *E. coli*. *In vivo*, the vast majority of DNA is negatively supercoiled, so unless the plasmid has an unusual secondary structure, it is likely negatively supercoiled when purified from the bacterial cells. Aliquots of the plasmid were then treated with purified enzyme, reverse gyrase, from the microbe *A. fulgidus* for varying amounts of time. When the plasmid is treated for a short time with reverse gyrase, a series of bands further up on the gel (compared to the 0 min treatment) appear. These correspond to the plasmid becoming *less* supercoiled, in this case, less negatively supercoiled. Each band is a different topoisomer, i.e. the same plasmid with a different number of supercoils. As the times get longer, the bands start to move back down the gel, indicating an increase in supercoiling. In the absence of evidence to the contrary, we assume that the DNA started out as negatively supercoiled. Therefore, the conclusion is

that reverse gyrase increases positive supercoiling, initially removing negative supercoils, and ultimately adding positive supercoils.

Image Descriptions

Figure 1 image description:

A DNA gel. Lanes indicate the amount of time the sample is treated with reverse gyrase. At $t = 0$ min, there is one band near the bottom of the gel. At $t = 0.25$ min, there are a series of bands further up the gel. The series of bands moves up the gel & then back down the gel until by $t = 3$ min nearly all the DNA is present in one band at the same position as the band present at $t = 0$ min. ↩

Thumbnail

"Gel electrophoresis 2.jpg" ↗ by Mnolf is licensed under [CC BY-SA 3.0](#) ↗.

Description: Gel electrophoresis of DNA made visible using ethidium bromide and ultraviolet light.

Author

Katherine Mattaini, Tufts University

1. McClendon, A. K., A. C. Rodriguez, and N. Osheroff. 2005. Human Topoisomerase II α Rapidly Relaxes Positively Supercoiled DNA: IMPLICATIONS FOR ENZYME ACTION AHEAD OF REPLICATION FORKS*. *Journal of Biological Chemistry* 280:39337–39345. ↩
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15: GFP Fusion Proteins

Summary

Fluorescent proteins like GFP can be fused to other proteins of interest to visually track the protein's location in living cells.

Also known as

GFP-tagged proteins, fluorescently-tagged proteins

Note: GFP variants are now available in every color of the rainbow, and they can also be used for this application.

Samples needed

In order to attach a GFP tag to a protein, a cDNA sequence encoding the protein must be available for molecular cloning.

Once the gene for the GFP-tagged protein is introduced into a cell or organism, the location can be visualized even in live cells. Cells can also be fixed and fluorescent signal will persist.

Method

In order to generate a GFP fusion protein, a cDNA that encodes the protein of interest must be available or obtainable. Then, through molecular cloning, the cDNA for GFP must be cloned upstream or downstream of the cDNA for the protein of interest, in frame, creating a fusion protein.

Next, the modified DNA must be introduced into the cell of interest. This can be done through a number of methods, either transient or stable transfection, [CRISPR](#), or others.

Finally, the GFP fusion protein can be tracked via fluorescent microscopy on live cells. The GFP fusion protein can also be tracked in multiple other ways, essentially by using the GFP portion of the protein in the same way as one would use an epitope tag. For instance, if a researcher wanted to [immunoprecipitate](#) the protein of interest, they could use an antibody against either the protein of interest, or against GFP.

Controls

GFP is not a small addition to a protein! Therefore, every effort should be made to determine whether the addition of the GFP to the protein of interest affects the protein's normal function.

Interpretation

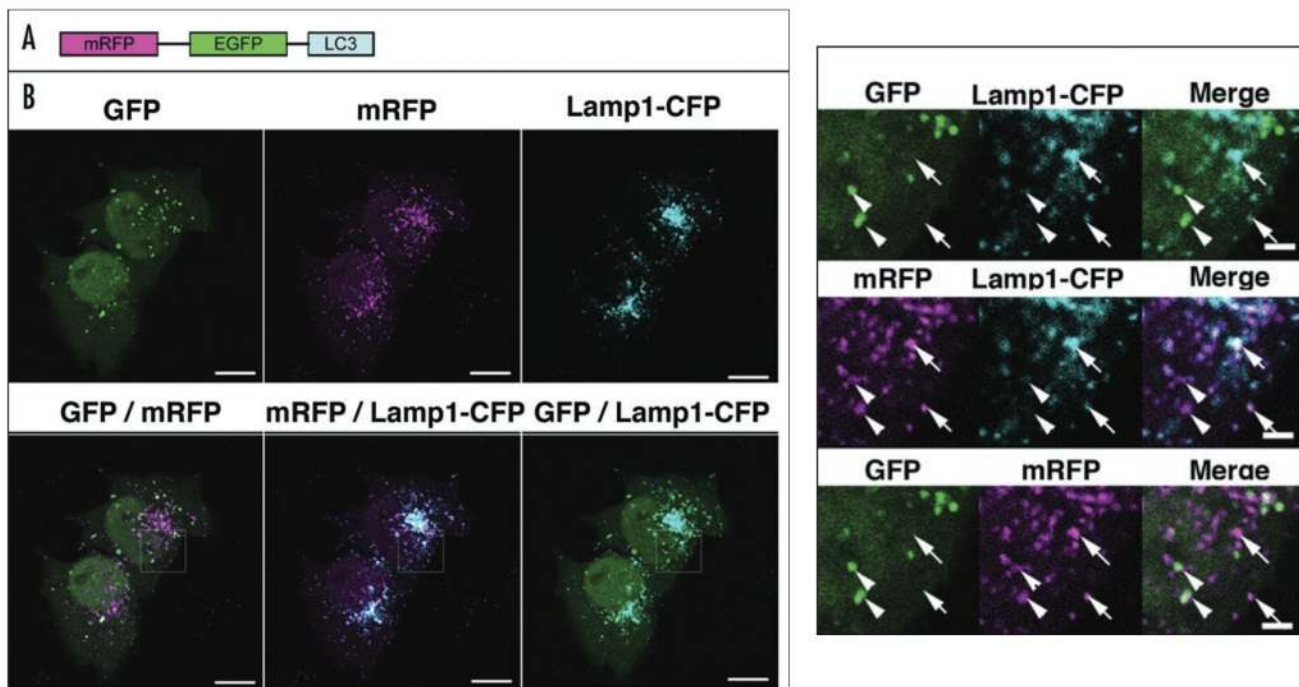


Figure 1. Confocal microscopy of HeLa cells transfected with various fluorescent protein fusions. Relevant section of caption for published figure reads: “The GFP and mRFP signals of tflc3 show different localization patterns. (A) Diagram of tflc3 structure. (B) HeLa cells were cotransfected with plasmids expressing either tflc3 or Lamp1-CFP. Twenty-four hours after the transfection, the cells were starved in Hanks’ solution for 2 hours, fixed and analyzed by microscopy. The lower panels are a higher magnification of the upper panels. Bar indicates 10 mm in the upper panels and 2 mm in the lower panels. Arrows in the lower panels point to typical examples of colocalized signals of mRFP and Lamp1. Arrowheads point to typical examples of colocalized particles of GFP and mRFP signals.” “Figure 4” by Kimura et al. ^[1] [\[Image description\]](#)

Table 1. Colocalization of mRFP and GFP signals of tflc3 with Lamp1, %. "Table 1" by Kimura et al. ^[1].

	mRFP	GFP	Lamp1
mRFP signals	N/A	24 ± 5	67 ± 12
GFP signals	86 ± 4.6	N/A	6.7 ± 2.6

Autophagy is a process by which starving cells engulf some of their cytoplasm within a double-membrane vesicle to recycle the contents in order to stave off cell death. The initial vesicle is called an autophagosome. Eventually, these vesicles fuse with lysosomes, and form the acidic autolysosome. In Kimura et al., the authors describe the development of a tool for interrogating the maturation pathway from autophagosomes to autolysosomes. This tool is a “tandem fluorescent” LC3 protein, the LC3 protein fused with *both* monomeric red fluorescent protein (mRFP) *and* green fluorescent protein (GFP). They call this tflc3. Before their study, it was already known that LC3 is associated with autophagosome membranes from the earliest stages of their formation. In the figure, we also see another fusion protein: Lamp1-CFP. CFP is cyan fluorescent protein, and Lamp1 is a protein found in the lysosome, and eventually the autolysosome.

Earlier in their study, Kimura et al. showed that mRFP-LC3 showed more puncta in cells than GFP-LC3 and hypothesized that this could be due to the less stable GFP denaturing in the acidic environment of the autolysosome, whereas mRFP remained stable there. To test this hypothesis, they tested co-localization of tflc3 (marks autophagosomes and autolysosomes) with Lamp1-CFP (marks lysosomes and autolysosomes). The figure above shows both the microscopy images and the quantification (Table 1) of co-localization from those images. Nearly all the GFP signal co-localized with mRFP signal, which is expected, since both signals come from the same fusion protein. However, a significant proportion of the mRFP signal does *not* co-localize with the GFP signal,

implying that some fraction of the fusion proteins have lost GFP signal but maintained mRFP signal, which is consistent with the authors' hypothesis that GFP was denaturing in a location where mRFP was still folded. They confirmed that this location was the autolysosome by showing that mRFP co-localized with Lamp1-CFP in locations where GFP signal was absent.

This is also a good example of why it's so important to test your assumption that your protein of interest is present exactly where you see a signal from your fusion protein! LC3 is present in the autolysosomes, but the GFP signal is lost. In this case, the authors did their due diligence and figured this out. In fact, they were actually able to exploit this characteristic to make a reporter protein, tfLC3, which can now be used as a useful tool to determine whether various experimental conditions affect the autophagosome, the autolysosome, or both.

Image Descriptions

Figure 1 image description:

Panel A: A schematic of a single fusion protein containing mRFP, GFP, and LC3.

Panel B: A series of fluorescent microscopy images. All show fluorescent signals in puncta spread around 2 cells. In all cases, some of the puncta co-localize, but not all. Images shown are: GFP, mRFP, Lamp1-CFP, GFP/mRFP merge, mRFP/Lamp1-CFP merge, GFP/Lamp1-CFP merge. There are also insets with greater magnification showing a few puncta that exhibit co-localization. Quantification of extent of co-localization in Table 1. ↩

Thumbnail

"Green fluorescent protein image of the mouse cochlea.jpg" ↗ by Atwiki111 is licensed under CC BY-SA 4.0 ↗.

Description: Green fluorescent protein image of the mouse cochlea.

Author

Katherine Mattaini, Tufts University

-
1. Kimura, S., T. Noda, and T. Yoshimori. 2007. Dissection of the Autophagosome Maturation Process by a Novel Reporter Protein, Tandem Fluorescent-Tagged LC3. *Autophagy* 3:452–460. ↩

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- 36: Western Blot by Katherine Mattaini is licensed CC BY-SA 4.0.

16: Immunofluorescence Microscopy

Summary

Immunofluorescence microscopy shows where in a cell a particular protein is located.

Also known as:

Immunofluorescence, IF

Samples needed

Fixed cells or tissue on a slide

Method

To stain a sample for immunofluorescence microscopy, samples are first fixed, then solubilized. Solubilizing creates holes in the plasma membrane to allow the antibodies to enter the sample. The sample is then blocked, often with milk protein, to discourage any non-specific binding of antibodies. Next, the sample is incubated in a primary antibody, which specifically binds to the protein of interest. Then, the sample is incubated in a secondary antibody, which binds to the primary antibody based on what species the primary antibody was raised in. (For instance, the secondary antibody might be something like anti-rabbit IgG.) The secondary antibody is chemically attached to a fluorescent molecule that allows detection with a fluorescence microscope.

Controls

Frequently, in addition to antibodies for the protein of interest, cells are also stained with DAPI. DAPI binds to DNA, thus marking the cell nucleus. When DNA-bound, DAPI fluoresces, emitting blue light.

Often times, other controls for IF are not shown in a published work. However, if a researcher is using new IF antibodies for the first time, several controls are useful. A positive control that contains the protein of interest tells the researcher if the antibody works well in IF and/or if the conditions used for staining are optimal. A control without the primary antibody ensures that the secondary antibody is binding specifically to the primary antibody and not other proteins. Finally, a control without the secondary antibody ensures that any observed signal is not autofluorescence of the cells or tissues.

Interpretation

A

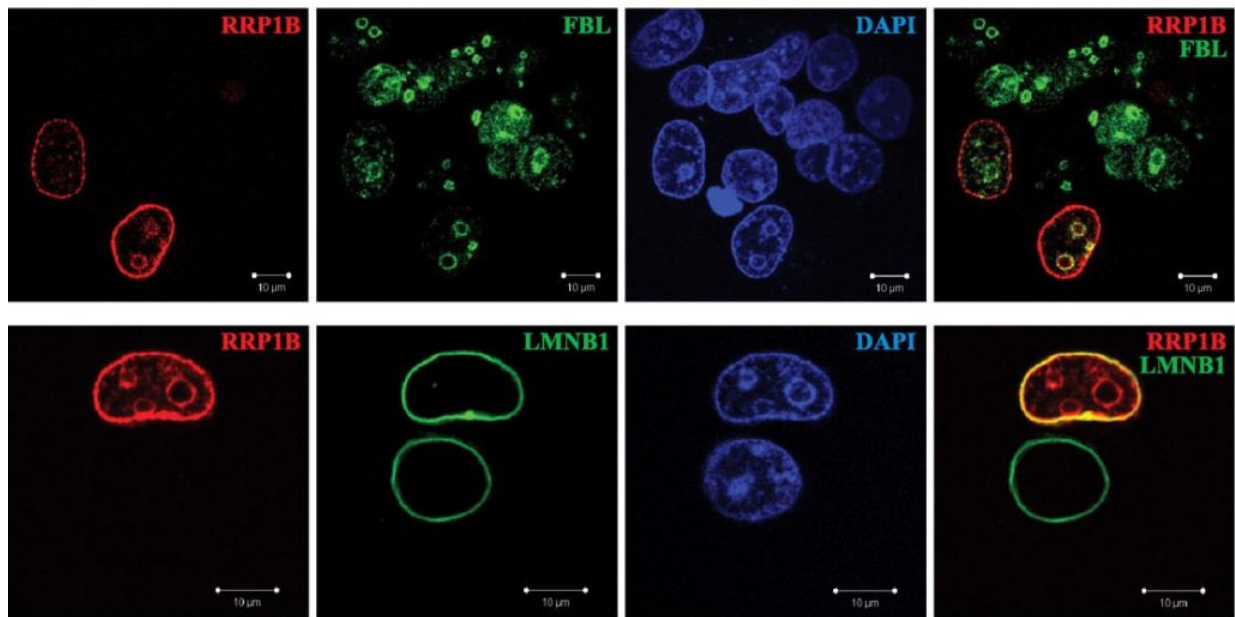


Figure 1. Two immunofluorescence experiments assessing intracellular localization of protein RRP1B. Relevant section of caption for published figure reads: “A, co-IF followed by confocal microscopy was performed to define the cellular localization of RRP1B. Hemagglutinin-tagged, full-length RRP1B was ectopically expressed in HeLa cells to define its cellular localization. Cells were co-stained for ectopic RRP1B (red), either the nucleolar marker fibrillarin (*FBL*; top) or the nuclear envelope marker lamin B1 (*LMNB1*; bottom) (green), and double-stranded DNA (*DAPI*; blue). ” “Figure 2” by Crawford et al.^[1] [Image description]

The reader can tell that these images focus on cell nuclei, because of the blue rounded structures in the DAPI channel. The authors are interested in the protein RRP1B and want to determine where it is within the cell. In the top panels, cells have been stained for both RRP1B and the protein fibrillarin, which is known to localize to the nucleolus. The top right panel is the overlay of the red (RRP1B) and green (fibrillarin) channels. In the image, locations with both red and green signals appear yellow, indicating that both proteins are found in that location. This is one piece of evidence that RRP1B is found in the perinucleolar region (region surrounding the nucleolus). The bottom panels are similar, but the green channel shows lamin B1, a protein that is known to localize to the nuclear envelope. The bottom right panel shows a nucleus with a yellow outline, demonstrating that RRP1B is also found at the periphery of the nucleus.

Note that in this experiment RRP1B was expressed ectopically, and the primary antibody detects the HA-tag on RRP1B, not RRP1B itself. Therefore, not all cells show the presence of HA-RRP1B. RRP1B may be present in other cells, but since it is the endogenous protein and not the tagged protein, it is not detected in this experiment. This is not considered ideal, but if an immunofluorescence antibody for the protein of interest is not available, this is one option to determine intracellular localization.

Image Descriptions

Figure 1 image description:

Two immunofluorescence experiments with four panels each. In both experiments, RRP1B (red) outlines the nucleus and nucleoli; DAPI (blue) stains throughout the nucleus. In the first experiment, FBL (green) marks the nucleoli; in the red/green overlay, the nucleoli are outlined in yellow. In the second experiment, LMNB1 (green) outlines the nucleus; in the red/green overlay, the nucleus is outlined in yellow. ↩

Thumbnail

"Spinal cord gray matter immunofluorescence staining, confocal imaging.png" ↗ by Wisstock is licensed under CC BY 4.0 ↗.

Description: Immunofluorescent staining of the gray matter in the rat spinal cord cross-section in a fixed specimen, resulting from a series of confocal images.

Author

Katherine Mattaini, Tufts University

-
1. Crawford, N. P. S., H. Yang, K. R. Mattaini, and K. W. Hunter. 2009. The metastasis efficiency modifier ribosomal RNA processing 1 homolog B (RRP1B) is a chromatin-associated factor. *Journal of Biological Chemistry* 284:28660-28673. [↩](#)
-

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17: Immunohistochemistry

Summary

Immunohistochemistry shows how much and where in a sample cell a particular protein is expressed. This technique is very similar to immunofluorescence microscopy, but detection is through visible color instead of fluorescence.

Also known as:

IHC

Samples needed

Fixed cells or tissue on a slide

Method

Many IHC samples come from a larger piece of tissue. Immediately upon collection, the tissue should be fixed, frequently in formalin. The tissue is embedded in wax, then sectioned, or cut into very, very thin slices, around a few μm thick. The section is mounted on a glass slide. When a researcher is ready to perform IHC, the wax is removed from the sample and antigen retrieval is performed, often by heating. The sample is then blocked, often with milk protein, to discourage any non-specific binding of antibodies, and also any endogenous peroxidase activity is blocked. Next, the sample is incubated in a primary antibody, which specifically binds to the protein of interest. Several different methods can be used at this point, but all result in a colored substance being deposited on the sample only at the locations where the protein of interest is located. Finally, the sample is counterstained with H&E.

Controls

Frequently, in addition to antibodies for the protein of interest, cells are also stained with hematoxylin and eosin (H&E stain). Hematoxylin stains nuclei purple, and eosin stains cytoplasm and extracellular matrix pink. Almost all histology samples, even those not subjected to IHC, are stained with H&E.

As for [IF](#), often times, other controls for IHC are not shown in a published work. However, if a researcher is using new IHC antibodies for the first time, several controls are useful. A positive control that contains the protein of interest tells the researcher if the antibody works well in IHC and/or if the conditions used for staining are optimal. A control without the primary antibody ensures that the secondary antibody is binding specifically to the primary antibody and not other proteins.

Thumbnail

"Kidney cd10 ihc.jpg"  by Nephron is licensed under [CC BY-SA 3.0](#) .

Description: Immunohistochemical staining of a non-neoplastic kidney with CD10 (Nephrlilysin).

Author

Katherine Mattaini, Tufts University

This page titled [17: Immunohistochemistry](#) is shared under a [CC BY-SA 4.0](#) license and was authored, remixed, and/or curated by [Katherine Mattaini](#).

18: In Situ Hybridization

Summary

In situ hybridization shows where in a tissue sample an RNA of a specific sequence is found or where in a set of chromosomes a DNA of a specific sequence is found.

Also known as

In situ, ISH

Caution

Usually when a specific DNA sequence (instead of RNA) is the target, this method is referred to as fluorescence *in situ* hybridization, or FISH. *Usually*, if someone refers to the method as just “*in situ*,” they mean they are probing for RNA, and if they say “FISH,” they mean they’re probing for DNA, but it’s best to clarify.

Samples needed

Fixed whole tissue, fixed tissue sections, or fixed cells

Method

Here, we will focus on how *in situ* is used to probe for an RNA sequence in a tissue sample, but the principles are the same across applications. First, the sample is chemically fixed, meaning that any living cells are killed and all metabolic activity ceases, and the tissue structure is chemically preserved. Next, the sample is permeabilized to allow the probe access to the nucleic acids in the sample. The sample is then incubated with a nucleic acid probe complementary to the RNA of interest. The probe binds the RNA transcript if present. The probe must be attached to another molecule that allows for visualization, whether that be a radioactive label, fluorescent label, etc.

Controls

The probe binding specificity should be tested through the use of positive and negative controls.

Interpretation

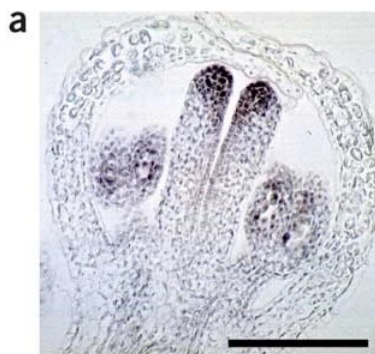


Figure 1. In situ hybridization for SPATULA mRNA. Relevant section of caption for published figure reads: “(a) Localization of SPATULA mRNA in a developing Arabidopsis flower (longitudinal section). Note the expression in the tips of the two carpels. Signal can also be seen in the anthers.” “Figure 1” by Brewer et al.^[1]. [\[Image description\]](#)

This image comes from a methods paper detailing how to perform *in situ* hybridization on *Arabidopsis* tissues, but a very similar photograph was generated in another study by some of the same researchers on *SPATULA* function^[2]. The study showed that *SPATULA* encodes a transcription factor that controls the growth of certain reproductive structures during flower development,

including the carpels, which produce female gametes, and anthers, which produce male gametes. One step in showing that *SPATULA* regulates the growth of these structures was demonstrating that *SPATULA* is expressed there. The *in situ* hybridization results in Figure 1 display staining in the relevant structures, indicating that *SPATULA* mRNA is present.

Image Descriptions

Figure 1 image description:

A stained longitudinal section of a developing flower. The tissue is shaped like a hollow bulb with two finger-like projections inside, and one more bulbous projection on either side of the fingers. The “fingers” are the carpels, and strong staining can be seen at the “fingertips.” The bulbs are anthers, which have lighter staining.

Thumbnail

"[Xenopus laevis embryo.jpg](#)" ↗ by Emily is licensed under [CC BY 4.0](#) ↗.

Description: *In situ* hybridization of MRF4 (myf6) gene in *X. laevis* embryo.

Author

Katherine Mattaini, Tufts University

1. Brewer, P. B., M. G. Heisler, J. Hejác̃ko, J. Friml, and E. Benková. 2006. *In situ* hybridization for mRNA detection in Arabidopsis tissue sections. *Nature Protocols* 1:1462–1467. ↩
 2. Heisler, M. G. B., A. Atkinson, Y. H. Bylstra, R. Walsh, and D. R. Smyth. 2001. *SPATULA*, a gene that controls development of carpel margin tissues in Arabidopsis, encodes a bHLH protein. *Development* 128:1089–1098. ↩
-

18: [In Situ Hybridization](#) is shared under a [CC BY-SA 4.0](#) license and was authored, remixed, and/or curated by LibreTexts.

- 36: [Western Blot](#) by [Katherine Mattaini](#) is licensed [CC BY-SA 4.0](#).

19: LC-MS

Summary

LC-MS is a method for separating mixtures of substances and identifying the components. In biological research, LC-MS is often used to identify the individual proteins present in a mixture.

Also known as

Liquid chromatography-mass spectrometry

Samples needed

In biology, most LC-MS is performed on a solution containing a mixture of proteins

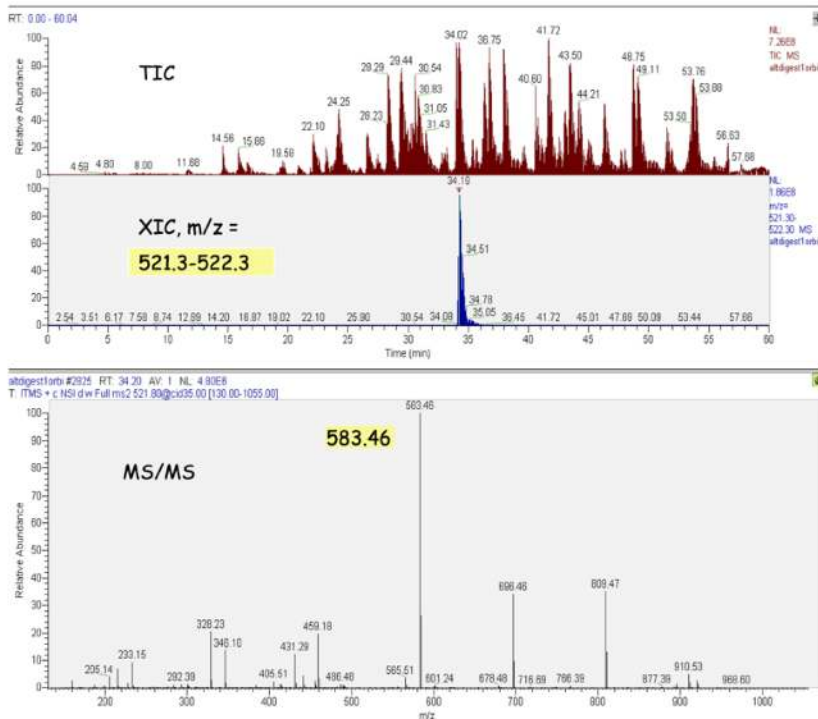
Method

The goal of an LC-MS experiment is to identify as many proteins as possible from a mixture. Often, the proteins are first digested with a protease into a complex mixture of peptides. Then the peptides are subjected to liquid chromatography, a type of [column chromatography](#), to separate the peptides so that only one or a few peptides at a time are eluted into the interface device. The type of column used determines which proteins enter the interface earlier vs. later.

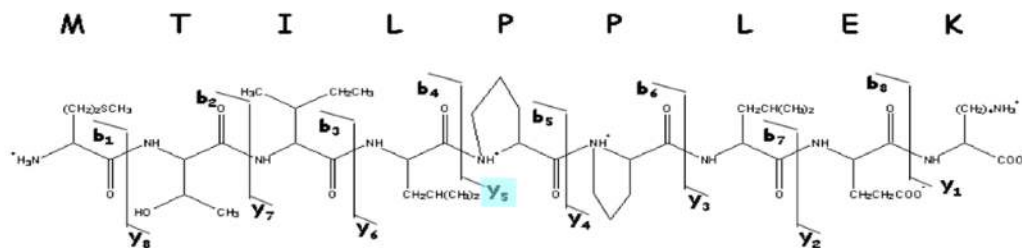
Mass spectrometry is an analytical method in which the mass-to-charge ratio (m/z) of ions can be determined. Therefore, peptides that enter the mass spec must be charged. If this is not already the case, it is achieved by one of several interface methods between the LC and the mass spec, including the popular electrospray ionization. The peptides then pass through a tandem mass spec, where the first device measures the m/z of the intact peptide (parent ion), the peptide is then fragmented, and the second mass spec determines the m/z of each fragment (daughter ions). The resulting spectra, called “peptide fingerprints,” are then computationally compared to a database of theoretical spectra of digest peptides of known proteins. Ultimately, this results in a list of protein “hits,” identifying the proteins that were present in the original mixture before digestion.

Interpretation

A



B



C

{MATRIX} Mascot Search Results

Peptide View

MS/MS Fragmentation of **MTILPPLEK**

Found in **ALAT1_HUMAN**, Alanine aminotransferase 1 (EC 2.6.1.2) (ALT1) (Glutamic-pyruvic transaminase 1) (GPT 1)

Match to Query 762: 1040.593484 from(521.304018,2+)

altdigest1orbi scan 2825 2825 (altdigest1orbi.2825.2.dta)

From data file K:\Orbitrap_62\Z05May2007\altdigest1orbi.mgf

Click mouse within plot area to zoom in by factor of two about that point

Or, Plot from 100 to 1000 Da

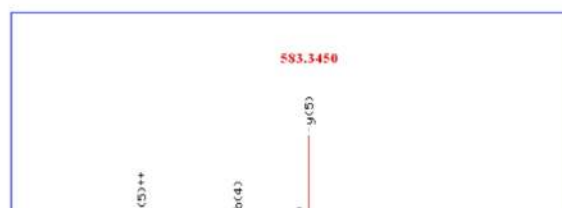
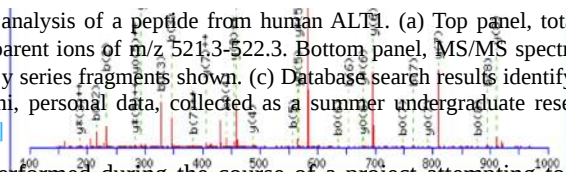


Figure 1. LC-MS analysis of a peptide from human ALT1. (a) Top panel, total ion chromatogram. Middle panel, extracted ion chromatogram of parent ions of m/z 521.3-522.3. Bottom panel, MS/MS spectrum of daughter ions. (b) Structure of ALT1 target peptide with b and y series fragments shown. (c) Database search results identifying spectrum from a as belonging to human ALT1 protein. K. Mattaini, personal data, collected as a summer undergraduate research fellow, 2007, Mayo Clinic, Rochester, MN. [\[Image description\]](#)



This experiment was performed during the course of a project attempting to develop an LC-MS/MS assay for the liver disease biomarker ALT1 in human serum. The top panel in a shows what is called a total ion chromatogram. This means that the y axis measures abundance of any peptides being eluted from the column over the course of the entire run. The middle panel in a is the extracted ion chromatogram. This means that the user has asked the program to show *only* ions of a particular m/z, in this case, 521.3-522.3. We see that a parent ion with this m/z was eluted between 34 and 35 minutes into the run. Finally, the bottom panel in a shows the MS/MS spectrum containing the daughter ions from the fragmentation of the parent ion. This result is what will be compared to the database. Panel b shows the peptide that makes up the parent ion in this case. In MS/MS, most peptides are fragmented at the peptide bonds, resulting in a series of y and b ions, shown in panel b. Note that the y5 ion (PPLEK) is highlighted in cyan. Panel c shows the results when the daughter ion spectrum from a was submitted to the Mascot tool. The tool identified the analyte from the panel a spectrum as the MTILPPLEK tryptic peptide from ALT1. The most abundant ion in both the theoretical and actual spectra (panels c and a, respectively,) is the y5 ion highlighted in panel b.

Image Descriptions

Figure 1 image description:

Panel a, top: A chromatogram, showing abundance on the y axis and time on the x axis. The chromatogram shows abundant peaks over the entire course of the 60-minute run, especially from 15 minutes onward.

Panel a, middle: A chromatogram, showing a single peak between 34 and 35 minutes. The label says “XIC, m/z = 521.3-522.3.”

Panel a, bottom: A mass spectrum, showing multiple peaks, with the most abundant peak at m/z = 583.46.

Panel b: The chemical structure of the peptide MTILPPLEK. At each peptide bond is a line, with a b ion on the left and a y ion on the right. The b1 ion is the first amino acid, M, and the b8 ion is the first 8 amino acids, MTILPPLE. The y1 ion is the last amino acid, K, and the y8 ion is the last 8 amino acids, TILPPLEK. The y5 ion, PPLEK, is highlighted.

Panel c: An image capture of a web page, Mascot search results. The results show a theoretical mass spectrum that closely matches the real spectrum from panel a. The peptide is identified as MTILPPLEK from ALAT1_HUMAN, alanine aminotransferase 1. [↩](#)

Thumbnail

"Mass spectrum brassicasterol.png" [↗](#) by Smmudge is licensed under [CC BY-SA 3.0](#) [↗](#).

Description: Mass spectrum of brassicasterol.

Author

Katherine Mattaini, Tufts University

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- 36: Western Blot by Katherine Mattaini is licensed [CC BY-SA 4.0](#).

20: Molecular Cloning

Summary

Molecular cloning refers to the isolation and amplification of a specific DNA sequence *in vivo*. It uses a host organism (usually bacteria) to produce a large number of identical copies of a recombinant DNA sequence that can be used for other molecular biology applications.

Also known as

Cloning

Samples needed

Concentrated solutions of a cloning vector (plasmid) and DNA containing the sequence to be cloned. The vector needs to contain an origin of replication specific to the organism into which the vector will be transformed, along with a gene encoding a selectable marker (often an antibiotic resistance gene).

Method

There are many methods for cloning a piece of DNA, but we will focus here on a classical molecular cloning process using restriction enzymes. The principles apply to most other cloning methods. You can see a diagram depicting the steps to be described in Figure 7.1 in [J.C. Lessard's chapter "Molecular Cloning."](#)^[1] First, the DNA to be cloned (called the insert) is obtained and isolated through PCR, DNA synthesis, or an alternative method. The vector and insert are then digested with restriction enzymes, producing ends that are complementary to each other. The digested DNA is purified. The digested vector and insert are incubated with DNA ligase and transformed into a host (usually bacteria). Host cells that take up the plasmid are selected for by plating on agar plates containing a chemical/compound that is toxic to cells lacking the plasmid. Cell colonies that grow on the plates can be expanded and the plasmid DNA can be purified from them. Restriction enzyme digestion (see interpretation), PCR, or DNA sequencing is often used to determine which plasmids contain the desired insert DNA.

Controls

Typically, a ligation reaction containing only the vector + DNA ligase is included, to make sure that bacterial colonies recovered after transformation don't contain a religated vector without an insert.

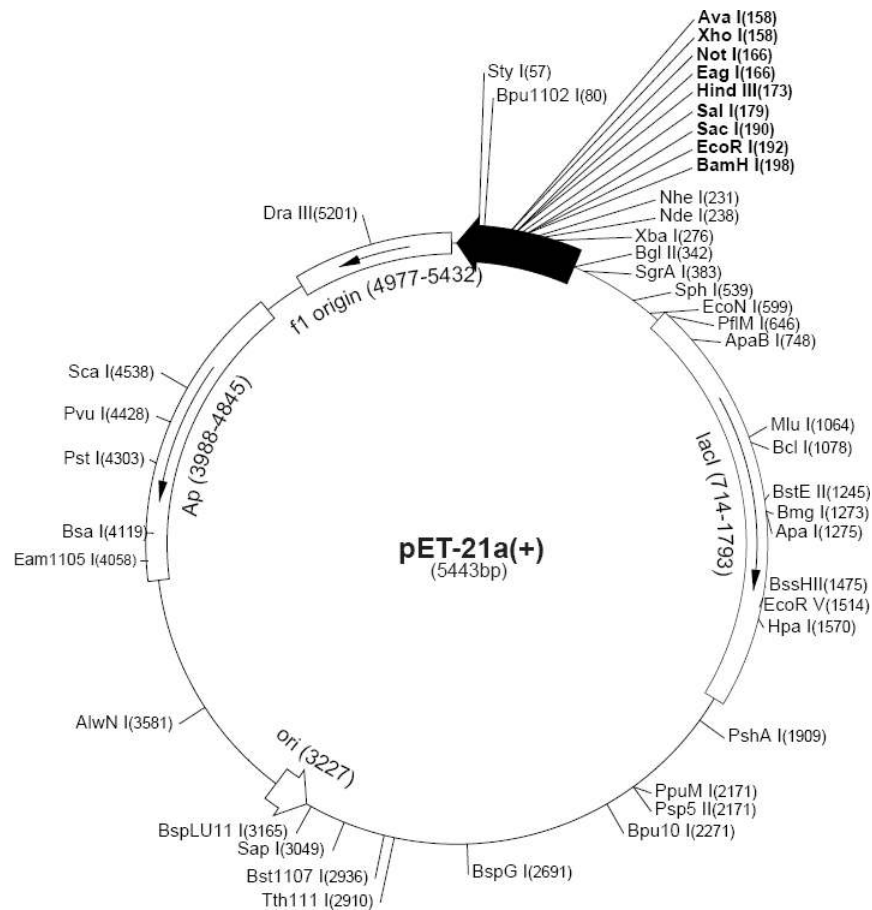


Figure 1. Map of the pET21a+ vector construct from [Addgene](#), accessed 9/4/04. [\[Image description.\]](#)

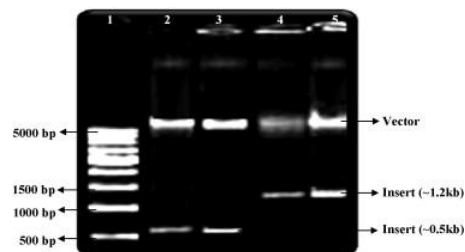


Figure 2. Confirmation of inserts within the pET21a+ vector constructs. Lane 1: 500 bp DNA ladder. Lanes 2-5: results of *Bam*HI and *Sal*I restriction enzyme digests of recombinant plasmids from OK-5 (lanes 2 and 3) and OM-6 (lanes 4 and 5) strains. Figure 5 from Gohal and Singh^[2]. [\[Image description.\]](#)

In this cloning experiment, alkaline protease genes from two haloalkaliphilic bacteria strains isolated in India (OK-5 and OM-6) were PCR amplified using genomic DNA from the bacteria as a template. The PCR products and the pET21a+ vector were both digested with *Bam*HI and *Sal*I restriction enzymes and the products were purified. Following ligation, transformation into *E. coli*, and plating on ampicillin-containing solid media, colonies were isolated, grown in ampicillin-containing liquid media, and plasmids were purified from each culture.

Purified plasmids were then digested with *Bam*HI and *Sal*I restriction enzymes and the products were separated by [gel electrophoresis](#). The results from lanes 2 and 3 show that the alkaline protease gene from the OK-5 strain was successfully cloned into pET21a+, while the results from lanes 4 and 5 show that the alkaline protease gene from the OM-6 strain was successfully cloned into the plasmid.

Image Descriptions

Figure 1 image description:

A map of the pET21a+ cloning vector. Unique cloning sites are indicated by lines labeled with the corresponding restriction enzyme recognition sequences. ↩

Figure 2 image description:

An agarose gel, stained with the intercalating agent ethidium bromide, showing the restriction enzyme digestion products of recombinant vectors created in a cloning experiment. Plasmids containing an insert produce two bands on the gel. ↩

Thumbnail

"BamHI.png" ↗ by Simon Caulton is licensed under [CC BY-SA 4.0](#) ↗.

Image description: Restriction of DNA by BamHI enzyme. The enzyme cuts the DNA at the sequence GGATCC and leaves 'sticky ends' that can attach to complementary sticky ends.

Author

Mitch McVey

Tufts University

-
1. J.C. Lessard. Molecular Cloning. Methods in Enzymology. 2013. 529: 85-98. ↩
 2. Gohel, S.D. and S.P. Singh. Cloning and expression of alkaline protease genes from two salt-tolerant alkaliphilic actinomycetes in *E. coli*. Int J Biol Macromol. 2012. 50:664-71. ↩
-

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21: Northern Blot and Southern Blot

Summary

Northern blot (RNA) and Southern blot (DNA) are methods used to detect the presence of a particular nucleic acid sequence in a mixture.

Also known as

No alternative names

Caution

Nomenclature note: The Southern blot is named after its inventor, Edward Southern, and therefore, the word “Southern” is a proper noun and should always be capitalized in its name. However, the northern blot and western blot were named in reference to the Southern blot and the cardinal directions. Therefore, the words “northern” and “western” in the method names are not proper nouns, and should *not* be capitalized.

Samples needed

Mixtures of nucleic acids

Method

The overall methods for northern and Southern blots are very similar, although they are used to detect different molecules, RNA and DNA, respectively. The first step is to separate the nucleic acid mixture by [gel electrophoresis](#). Following electrophoresis, the nucleic acids are transferred to a membrane, then the membrane is treated with a single-stranded nucleic acid probe. If using double-stranded nucleic acid samples, a denaturing step is required to expose the nitrogenous bases.

The probe has two key features. First, it is able to base pair with the DNA or RNA sequence of interest. Second, it is labeled for visualization, often either by a radioactive or fluorescent molecule. When the membrane is visualized, the signal indicates the size (due to the electrophoresis step), and to some extent, the amount of the nucleic acid sequence of interest.

Controls

Ideally, there should be positive and negative controls to test the specificity of probe binding. The electrophoresis step should also include molecular weight ladders.

Interpretation

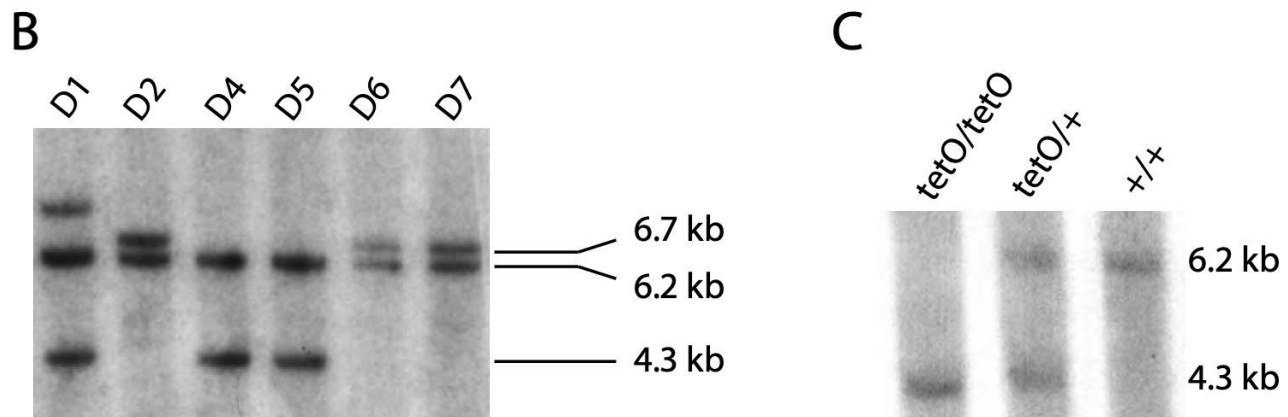


Figure 1. Southern blot results. Relevant section of caption for published figure reads: “**(B)** Southern blot analysis of SpeI-digested genomic DNA from six *PHGDH*^{tetO}-targeted ES cells. Clones D4 and D5 exhibit proper targeting of the *Col1A* locus and an unaffected wild-type allele. **(C)** Southern blot analysis of SpeI-digested genomic DNA from mice of the indicated genotypes. ” “Figure S1” by Mattaini et al.^[1]. [\[Image description\]](#)

Figure 1 above shows the results of a two Southern blots, the first from embryonic stem cells that the authors were attempting to genetically modify, and the second from the resulting genetically modified mice. Importantly, the ES cells being used already had a modification that allowed researchers to easily insert a gene of interest. Therefore, when the genomic DNA was cut with the restriction enzyme SpeI, three band sizes were possible. The wild type locus is shown by the 6.2 kb band, the initial modification (to allow a transgene to be inserted) is shown by a 6.7 kb band, and the locus with the gene of interest (*PHGDH*) successfully inserted is shown by a 4.3 kb band.

In panel B, ES cell clones D2, D6, and D7 show one wild type locus and one modified locus without *PHGDH* (bands at 6.2 and 6.7 kb). Clones D4 and D5 show one wild type locus and one locus with *PHGDH* (bands at 6.2 and 4.3 kb). Clone D1 has a band at an unexpected size, so that would not be a good clone to select to use to make genetically modified mice. Panel C shows that, as expected, mice that have been PCR genotyped as having two copies of *PHGDH* (“*tetO/tetO*”) have only the 4.2 kb band, mice with no transgene (“*+/+*”) have only the 6.2 kb band, and mice with one copy of *PHGDH* (“*tetO/+*”) have both bands.

Note that when all of the DNA in a mouse cell is digested with SpeI, a huge smear of DNA would be seen on a DNA gel if a normal gel visualization method like ethidium bromide were used. The use of a probe complementary to the locus of interest allows the researchers to see only the bands they care about, not every single SpeI fragment.

Image Descriptions

Figure 1 image description:

Two Southern blots.

Results of first Southern blot

	D1	D2	D4	D5	D6	D7
Band >6.7 kb	x					
6.7 kb		x			x	x
6.2 kb	x	x	x	x	x	x
4.3 kb	x		x	x		

Results of second Southern blot

	tetO/tetO	tetO/+	+/+
6.2 kb		x	x
4.3 kb	x	x	

↩

Thumbnail

"[Southern blot membrane.jpg](#)" ↗ by Bojan Žunar is licensed under [CC BY-SA 4.0](#) ↗.

Description: Southern blot membrane.

Author

Katherine Mattaini, Tufts University

-
1. Mattaini, K. R., M. R. Sullivan, A. N. Lau, B. P. Fiske, R. T. Bronson, and M. G. Vander Heiden. 2019. Increased PHGDH expression promotes aberrant melanin accumulation. BMC Cancer 19:723. ↩
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22: ONPG Assay

Summary

The ONPG assay is an enzyme activity assay used to measure the activity of the enzyme β -galactosidase (β -gal). The breakdown of ONPG by β -gal results in a color change that can be measured and equated to the activity of β -gal.

Note

The ONPG assay is one of many enzyme activity assays. Enzyme activity assays can be used to measure the activity of a variety of different enzymes and activity measurements can be made using a variety of techniques including colorimetric assays, fluorescence assays, and radioactive assays.

Also known as

O-nitrophenyl β -d-galactopyranoside (ONPG) assay, β -gal activity assay, enzyme activity assay

Samples needed

Cell lysate containing β -gal or purified β -gal enzyme

Method

O-nitrophenyl β -d-galactopyranoside (ONPG) is a synthetic compound that is similar in structure to lactose, the natural substrate of β -gal. β -gal can use ONPG as a substrate and will cleave ONPG to produce galactose and o-nitrophenol. O-nitrophenol has a yellow color and results in a yellow color change.

In general, cell lysate or purified β -gal enzyme is added to a buffer containing ONPG and incubated to allow for ONPG to be able to interact with the β -gal enzyme. Following incubation for the desired time, the reaction is stopped by the addition of a basic solution (typically 1M Na₂CO₃) to change the pH and halt enzyme activity. Any visible color change can then be observed by eye. This color change can then be measured by a plate reader or spectrophotometer by measuring the absorbance at a wavelength of 405-420 nm.

Controls

Negative control lacking the enzyme or lacking the necessary enzyme substrate can be included. When assaying the activity of β -gal expressed by the *lacZ* gene on a plasmid, there is the possibility that endogenous *lacZ* in the chromosome is also producing β -gal. In this case an endogenous control (cells not containing the plasmid expressing *lacZ*) should also be included to control for the level of endogenous β -gal activity. A positive control, often purified β -gal enzyme, should also be included.

Interpretation



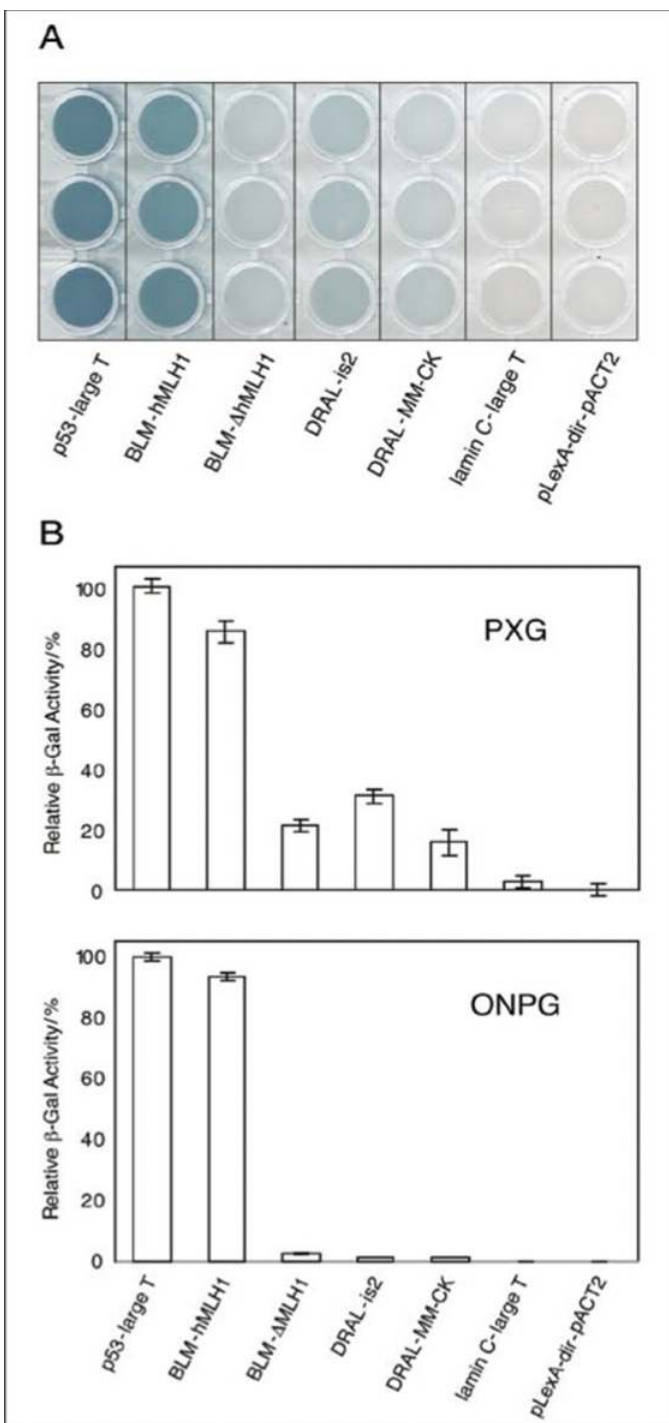


Figure 1. Measurement of protein interactions by pellet X-gal (PXG) and ONPG activity assays. Relevant section of caption for published figure reads “(A) Scanned image of a PXG assay in a 96-well microtiter plate after 30 min of incubation. Three independent transformants were assayed per protein interaction pair. (B) Relative β -gal activity in the PXG and ONPG assays. The p53-large T interaction was set arbitrarily to 100% for both assays. Error bars represent the standard deviation from three independent experiments in both assays.” “Figure 1” by Möckli and Auerbach^[1]. [\[Image description\]](#)

The authors wanted to assess if different proteins (p53, large T antigen, hMLH1, truncated hMLH1 (Δ hMLH1), DRAL, MM-CK, and is2) interacted with each other, to do this they utilized a yeast two-hybrid assay. In a yeast two-hybrid assay a yeast transcription factor is split into two domains, and these domains are then fused to protein pairs. Only if the two proteins interact will the full transcription factor be reconstituted and be able to promote a transcription of a reporter gene. In this assay, the *lacZ*

gene encoding for the β -gal enzyme is used as the reporter gene, meaning that the higher β -gal enzyme activity, the stronger the interaction between the two proteins tested.

Panel A shows an image of a 96-well plate where cell lysate containing the two proteins of interest have been incubated with X-gal, which is structurally similar to lactose and can serve as a substrate for β -gal. When X-gal is cleaved by the β -gal enzyme it results in a blue color change. Thus, the stronger the blue color change, the higher the β -gal activity. The authors also performed an ONPG assay to measure β -gal activity by measuring the yellow color change that results from cleavage of the ONPG substrate (not shown). Panel B shows the graphs of the β -gal activity measured by the PXG and ONPG assays. β -gal activity is shown relative to the activity from the p53/large T condition, as these proteins were previously shown to interact and should therefore result in β -gal activity.

Based on the measurements of β -gal activity from the PXG and ONPG assays the authors found the p53/large T interaction was the strongest and the DRAL/MM-CK interaction was the weakest. No β -gal activity was observed in the negative control co-expressing large T antigen and lamin C (that are not known to interact) or empty vectors only (pLexA-dir/ pACT2).

Image Descriptions

Figure 1 image description:

Panel A is an image of a 3 row x 7 column section of a 96-well plate. Each column is three replicates for a protein interaction in a PXG assay. From left to right the columns show: bright blue wells for the p53/large T condition, bright blue wells for the hMLH1/BLM condition, faint blue wells for the BLM- Δ hMLH1 condition, mild blue wells for the DRAL/FHL-2-is2 condition, faint blue wells for the DRAL/MM-CK condition, and clear wells for both the large T antigen/lamin C condition and the empty vector condition.

Panel B is two bar graphs of the relative β -gal activity. The top bar graph is the result of the PXG assay with p53/large T condition at 100% relative activity, the hMLH1/BLM condition at 85%, the BLM- Δ hMLH1 condition at 20%, the DRAL/is2 condition at 35%, the DRAL/MM-CK condition at 18%, and both the large T antigen/lamin C condition and the empty vector condition at around 0%. The bottom bar graph is the results of the ONPG assay with p53/large T condition at 100% relative activity, the hMLH1/BLM condition at 90%, and the rest of the conditions at 2-0%. ↩

Thumbnail

[Reaksi_ONPG.jpg](#) ↗ by BP63Vincent is licensed under [CC BY-SA 3.0](#) ↗.

Description: Chemical reaction showing ONPG broken down into galactose and o-nitrophenol (yellow).

Author

Rachel Ende, Tufts University

-
1. Möckli, N. and D. Auerbach. 2004. Quantitative β -Galactosidase Assay Suitable for High-Throughput Applications in the Yeast Two-Hybrid System. *BioTechniques* 36 (5): 872–76. doi:10.2144/04365PT03. ↩

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23: PCR

Summary

The polymerase chain reaction (PCR) is a method that amplifies a target DNA sequence.

Also known as

Polymerase chain reaction

Samples needed

Sample of DNA, which can be a mixture of sequences

Method

PCR is used to amplify, or make many additional copies of, a specific DNA sequence. PCR is used as a step in a wide variety of applications.

In order to perform a PCR, the following components must be included in the reaction mixture:

- A DNA sample containing the template DNA, i.e. the DNA sequence to be amplified
- A DNA polymerase enzyme
- A mixture of the four dNTPs
- Primers
- An appropriate buffer solution

The sequence amplified is determined by the use of primers: short oligonucleotides that anneal to specific DNA sequences. The DNA polymerase enzyme requires a primer to begin copying a DNA template, so it will only act on the 3' end of the primer or primers provided by the researcher.

The reaction mixture is then subjected to a series of different temperatures, usually in a machine called a thermal cycler. Each cycle of PCR should double the amount of target DNA sequence present. Each cycle consists of three steps:

Denature template (separate DNA strands) at high temperature: mid 90s °C

Anneal primers at low temperature: Exact temperature depends on primers, but usually in 50s °C

Extend by DNA polymerase at moderate temperature: often 72 °C

Controls

Sometimes primers will anneal to a DNA sequence that is not an exact match, and therefore, the amplified DNA will not be the desired sequence. A DNA sample without the desired template DNA can be used as a negative control to confirm primer specificity.

Furthermore, sometimes a no template control (water instead of DNA sample) is used to ensure that none of the other reagents are contaminated with template DNA

Interpretation

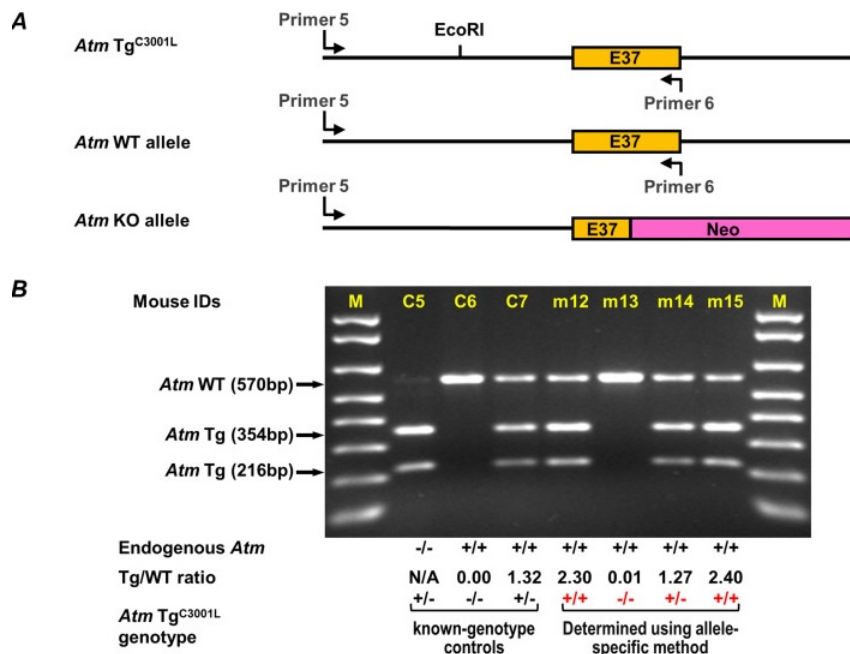


Figure 1. Genotyping of

transgenic mice by PCR, restriction enzyme digest, and gel electrophoresis. Relevant section of caption for published figure reads: “(A) Schematic illustration of *Atm* TgC3001L transgene, *Atm* WT allele, and *Atm* KO allele. Their sequences are aligned in reference to exon 37 (E37). Primers 5 and 6 are used to perform PCR, amplifying a DNA segment in both *Atm* TgC3001L transgene and *Atm* WT allele, but not in *Atm* KO allele. (B) The working principle of the allele-specific genotyping method. Mice with known genotype of transgene were used as controls (C5 to C7). DNA fragments from *Eco*RI digestion were analyzed in a 1.4% agarose gel. Below the gel are listed the genotype of *Atm* WT allele, the ratio of band intensities between Tg and WT alleles (Tg/WT ratio), and the genotype of *Atm* TgC3001L transgene for control (black) and assayed mice (assay-determined genotype is in red color, m12–m15).” “Figure 4” by Yang et al.^[1]. [\[Image description\]](#)

In this experiment, researchers tackled the problem of genotyping transgenic mice with a mutant *Atm* (C3001L) allele integrated into a random genomic locus, i.e. an *Atm* transgene. To fully determine the *Atm* status of these mice, researchers need to know **if each individual mouse has 0, 1, or 2 copies of the transgene** (“transgene zygosity”), as well as whether the endogenous *Atm* locus contains 0, 1, or 2 copies of an *Atm* null allele. When referring to the endogenous *Atm* locus, “+” denotes wild type and “-” denotes the null allele. When referring to the transgene (Tg), “+/+” denotes two transgenes, “+/-” denotes one, and “-/-” denotes none.

Panel A in this figure shows the reader where the two primers bind, and therefore, which segment of DNA will be amplified during PCR: the section between primers 5 and 6. The three possible alleles are shown: the *Atm* transgene with a C3001L mutation, wild type *Atm* at the endogenous locus, and an *Atm* null allele, also at the endogenous locus. The amplicons of WT and Tg are the same size, so a further step must be taken to distinguish them by gel electrophoresis. Primer 6 does not bind to the null allele, so no amplification will occur from that template.

Anticipating the problem of distinguishing the WT endogenous *Atm* from the transgene, the researchers introduced an *Eco*RI restriction enzyme site into an intron in the transgene amplicon. To genotype mice, two steps are required. First, an experiment **not shown here** is used to determine the *Atm* genotype at the endogenous locus. Second, to determine how many copies of the transgene are present, PCR is performed with primers 5 and 6, followed by an *Eco*RI digest and [gel electrophoresis](#). The number of transgene copies present is determined by comparing the intensity of the transgene bands to the WT band.

The results of such a genotyping experiment are shown in panel B. Remember that the genotype at the endogenous *Atm* locus is already known before the experiment shown. If the C3001L allele is present, two restriction fragments of 354 and 216 bp are expected due to *Eco*RI digestion. If the wild type allele is present, an intact amplicon of 570 bp is expected, as it lacks an *Eco*RI restriction site.

The number of transgenes can be determined by comparing the intensity of the band at 570 bp (WT) to the intensity of the bands at 354 & 216 bp (Tg). Consider control mouse C7. Researchers knew that it was +/+ at the endogenous locus and +/- at the transgene locus, and the Tg/WT ratio (for band intensity) was 1.32. All mice in this experiment except C5 are +/+ at the endogenous locus.

Therefore, any unknown mouse with a Tg/WT ratio of ~ 1.32 is +/- at the transgene locus, as seen for instance in m14. Mice that are +/+ at the transgene locus will have a ratio roughly double that, as seen in m12 and m15. Any mice that are -/- at the transgene locus will lack the smaller bands entirely, as we see in m13. (To determine transgene zygosity in mice -/- at the endogenous locus the researchers used a different reverse primer that could bind the null allele.)

Image Descriptions

Figure 1 image description:

Panel A: A schematic of the genomic DNA of two *Atm* alleles: *Atm* TgC3001L and *Atm* WT. Both alleles show primer 5 (upstream) and primer 6 (downstream) pointing toward one another. Primer 5 binds in an intron, and primer 6 binds in exon 37. The amplicon contains an *EcoRI* restriction site only in the mutant allele.

Panel B: An image of DNA gel.

Summary of results on gel in Figure 1, panel b.

Mouse	Endogenous <i>Atm</i> genotype	570 bp band present? (Indicates WT)	354 & 216 bp bands present? (Indicates Tg)	Tg/WT ratio	Sample type (Referring to <i>Atm</i> Tg)	<i>Atm</i> genotype	Tg
C5	-/-	N	Y	N/A	Known-genotype control	+/-	
C6	+/+	Y	N	0.00	Known-genotype control	-/-	
C7	+/+	Y	Y	1.32	Known-genotype control	+/-	
m12	+/+	Y	Y	2.30	Unknown transgene zygosity	+/+	
m13	+/+	Y	N	0.01	Unknown transgene zygosity	-/-	
m14	+/+	Y	Y	1.27	Unknown transgene zygosity	+/-	
m15	+/+	Y	Y	2.40	Unknown transgene zygosity	+/+	

↩

Thumbnail

"PCR tubes.png" ↗ by Madprime is licensed under [CC BY 1.0](#) ↗.

Description: Photo of a strip of PCR tubes, each tube contains a 1000uL (1mL) reaction.

Author

Katherine Mattaini, Tufts University

1. Yang, J., A. N. DeVore, D. A. Fu, M. M. Spicer, M. Guo, S. G. Thompson, K. E. Ahlers-Dannen, F. Polato, A. Nussenzweig, and R. A. Fisher. 2023. Rapid and precise genotyping of transgene zygosity in mice using an allele-specific method. *Life Science Alliance* 6: e202201729. ↩

23: PCR is shared under a [CC BY-SA 4.0](#) license and was authored, remixed, and/or curated by LibreTexts.

- 36: Western Blot by [Katherine Mattaini](#) is licensed [CC BY-SA 4.0](#).

24: pET Expression System

Summary

The pET system is a protein expression system developed by Novagen Inc. to express a protein of interest in special strains of *E. coli* upon treatment with the chemical IPTG (isopropyl- β -D-thiogalactoside).

Also known as

Can be used as a type of heterologous expression system, wherein a protein from one organism is expressed in a different organism.

Samples needed

Expression systems require two components:

1. A plasmid vector carrying the gene (or cDNA) of interest. In this case, that is a pET vector.
2. The cells to express the gene/cDNA. In this case, those are *E. coli* BL21 (DE3) cells.

Method

Heterologous expression systems using bacteria

One of the best reasons to transfer foreign DNA into another organism is to have the gene product expressed. Heterologous expression systems offer several advantages:

- High yields of normally scarce proteins can be obtained through over-expression.
- Proteins derived from organisms that are difficult to obtain, slow to grow, or dangerous to handle can be expressed in high yield in simple, safe, and cheap culture systems.
- Proteins for which no known purification schemes exist can be obtained as preponderant components of cultures or extracts.

In fact, even if the gene product of a particular gene is unknown, the gene can be expressed and its product studied by expression in a heterologous system.

E. coli has several advantages as an expression host. It is cheap and easy to grow in large quantity. Also, many well-studied bacterial promoters can be included in this system to drive expression of the cloned genes by known inducers. Expression of eukaryotic genes in a prokaryote, however, has some major disadvantages. These include the failure of prokaryotic cells to make appropriate post-translational modifications for eukaryotic proteins. This means that the induced proteins may not function correctly if their biological activity depends on correct glycosylation, disulfide bond formation, phosphorylation, or proteolytic processing.

Another disadvantage of prokaryotic expression systems is the inability of bacteria to transcribe correct mRNAs from cloned eukaryotic genes containing introns. Message processing systems that will correctly splice out intervening sequences are usually restricted to eukaryotes.

Bacterial expression systems can be used to produce either intact native proteins or **fusion proteins**. Native proteins require that the foreign gene be cloned downstream of a strong bacterial promoter and ribosome-binding site. Fusion proteins are exactly what their name implies: they are proteins that contain some amino acid sequence from two different proteins. Often, fusion proteins include the entire protein of interest and a short sequence from another protein, called an affinity tag, an epitope tag, or simply a tag. Proteins can be tagged on either the N- or C-terminus depending on the relative positions of the transcription start site, the coding sequences for the protein of interest and tag, and the stop codon. When **cloning** to create a tagged protein, it is imperative that the tag is in frame with the protein of interest.

Both types of expression system have advantages. Tagged proteins can be isolated easily even if you know nothing about the product of the cloned gene. Since part of the fusion protein consists of a known peptide tag, you can isolate the fusion protein using, for instance, **antibodies to the tag**. Also, fusion proteins are frequently more stable (less readily degraded) than native proteins expressed in the same host.

On the other hand, native proteins may fold in a conformation that more closely resembles the physiological protein structure. This would be important for enzymes or proteins with specific binding properties. Tags can also be problematic if the purified protein is being used to raise antibodies against the protein of interest. To raise polyclonal antibodies for lab use, the purified protein is injected into an animal (such as a mouse, rat, or rabbit), and the animal's immune system produces the antibodies. However, the tag is often more immunogenic than the protein of interest itself, which would result in too many anti-tag antibodies and not enough antibodies specific to the protein of interest. Therefore, a tag is often cleaved from the purified protein before using the protein to generate antibodies.

The pET expression system

The pET system was published by Studier and Moffatt in 1986¹ and further developed by Novagen². Expression systems have two components – the DNA vector and the host cells.

Vector

Here, we will discuss the specific vector pET28b as an example. Some important features of [the vector \(opens PDF of vector map from Millipore Sigma ↗\)](#):

- A **multiple cloning site (MCS)** that allows the researcher to easily insert a gene or cDNA of interest using one of several restriction enzyme cut sites.
- Regions encoding **epitope tags**. The pET28b vector allows the researcher to incorporate N-terminal polyhistidine (His) and T7 tags and/or a C-terminal His tag.

The pET28b plasmid has been engineered such that foreign genes are cloned into a site just downstream of an ATG initiation codon. Shortly after the initiation codon (but still 5' to the cloned gene) comes a series of six triplets in the DNA sequence that code for histidine residues. Upon translation, this polyhistidine tag will form part of the amino terminus of the protein expressed from this cloned gene. The expressed protein is, therefore, a kind of fusion protein, with a short peptide fused to the protein of interest.

The polyhistidine tag is useful for purification of the fusion protein. Polyhistidine binds with relatively high affinity to divalent cations, particularly Ni^{2+} , Co^{2+} , and Zn^{2+} . These cations can be "immobilized" on a resin, and the complex can be used to purify the recombinant protein based on the affinity of the polyhistidine tag for the divalent cations. This is one form of affinity chromatography, a standard technique useful for protein purification.

T7 is an epitope tag derived from the T7 major capsid protein with the amino acid sequence MASMTGGQQMG. The T7 tag can be recognized by a specific antibody, allowing detection and purification of pET28b expressed products by a second method (unrelated to the polyhistidine tag).

Lastly, there is a second polyhistidine tag in pET28b *downstream* of the cloning site. This can be used to create a C-terminally His-tagged fusion protein if the stop codon is left out of the coding sequence for the cloned gene and reading frame is maintained. If the stop codon is left in the coding sequence of the cloned gene, the C-terminal His tag is not translated.

- A **thrombin cleavage site**. The amino acid sequence following the tag contains a 6-residue stretch (L-V-P-R-G-S) that is the recognition site for a protease known as thrombin. Thrombin can be used to enzymatically cleave off the N-terminal His tag after purification if the tag's presence would impede downstream applications.
- A **promoter** derived from bacteriophage T7. Genes or cDNAs are cloned into the plasmid next to this promoter and therefore are under its control. The result is that cloned genes cannot be transcribed by the normal bacterial RNA polymerase, but only by T7 RNA polymerase.
- An **origin of replication**. This allows the plasmid to be replicated by the *E. coli* host machinery, independently of the chromosomal DNA.

- A **kanamycin resistance gene** (Kan^R). This encodes the enzyme NPTII (neomycin phosphotransferase II), which inactivates kanamycin³, making any cells expressing the gene resistant to this antibiotic.
- A *lacI* gene, encoding the **lac repressor** protein. The lac repressor is able to bind to a sequence from the lac operon, repressing its transcription. In the pET system, inclusion of *lacI* allows the researcher to control when the *E. coli* express the protein of interest (see below).

Cells & protein expression

Molecular biologists can **transform** a plasmid into *E. coli* to achieve different goals, and they need to use the right *E. coli* strain for the intended job. For instance, strains like JM109 *E. coli*, have good characteristics for cloning. However, to use *E. coli* to produce protein with the pET system, BL21(DE3) cells are used, which have been genetically modified for this purpose.

These cells have had the **T7 RNA polymerase gene** cloned into their chromosomal DNA (signified by the (DE3) designation) (Figure 2). This chromosomal copy of the T7 RNA polymerase gene has been placed under the control of a promoter known as *lacUV5*. This promoter, like the normal lac operon promoter in wild type *E. coli*, is repressed by the lac repressor protein and induced (released from repression) by lactose. Thus, T7 RNA polymerase is not transcribed in most situations, and therefore, the gene introduced in the pET28b plasmid is also not transcribed. Administering lactose (or its analogues) can "turn on" the expression of T7 RNA polymerase by causing a conformational change that renders the lac repressor protein inactive. Traditionally, isopropyl- β -D-thiogalactoside (IPTG) is used to induce expression because it binds to the repressor protein as lactose does, but cannot be hydrolyzed by the *lacZ* product β -galactosidase.

Once T7 RNA polymerase expression has been induced and T7 RNA polymerase synthesized by the bacterial cells, the gene or cDNA that was cloned into the plasmid vector (under control of the T7 promoter) can be transcribed. This allows the researcher to control timing of expression of the cloned gene.

When BL21 *E. coli* cells transformed with a pET plasmid are treated with IPTG, we say call it an **induction**, because the researcher is *inducing* expression of the gene of interest.

E. coli BL21(DE3)

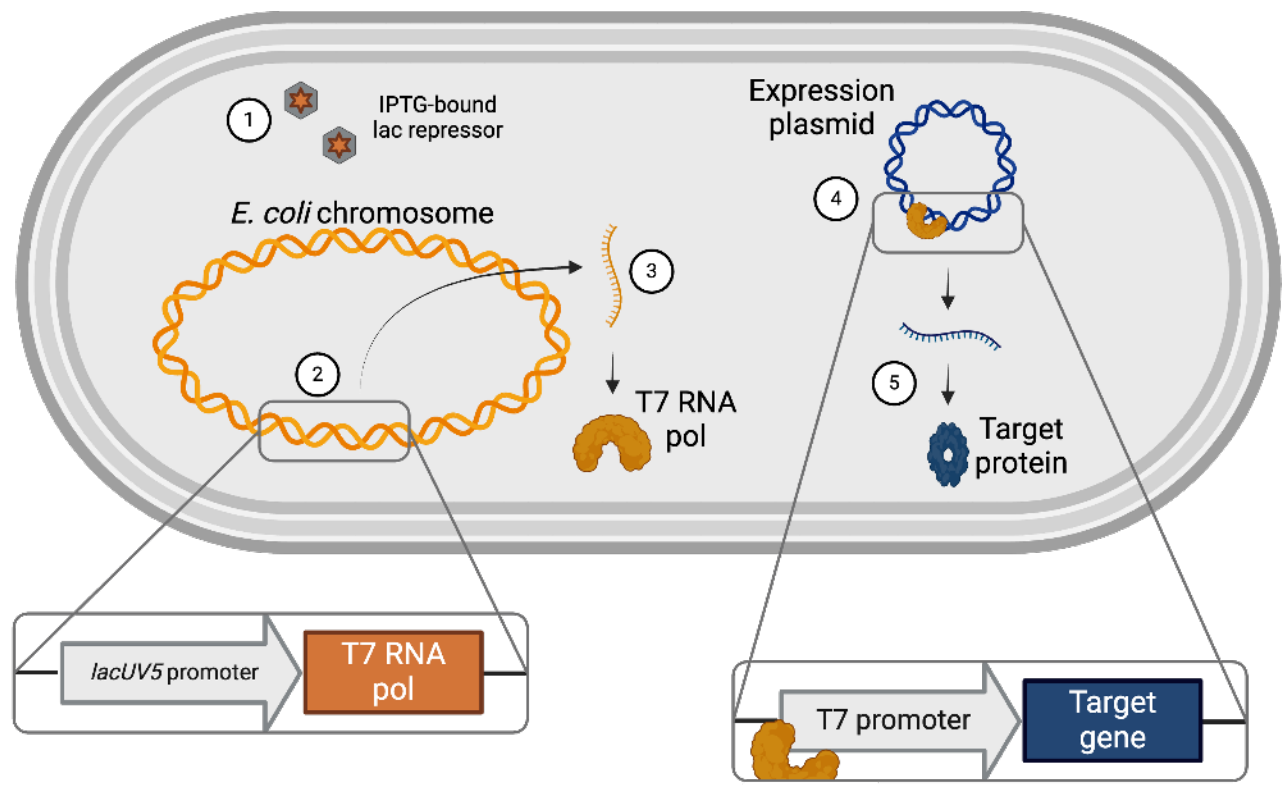


Figure 1. Overview of the pET expression system. Steps to express the cloned gene: (1) IPTG is administered and binds to the lac repressor protein, rendering it inactive. In the absence of IPTG, the lac repressor protein prevents expression of the T7 RNA pol. (2) BL21(DE3) *E. coli* contain a chromosomal copy of the T7 RNA polymerase gene under the control of the *lacUV5* promoter. (3) With the lac repressor protein no longer bound to the *lacUV5* promoter, *E. coli* cells' usual transcriptional and translational machinery produce T7 RNA polymerase mRNA and protein (orange/left). (4) T7 RNA polymerase binds to the T7 lac promoter in the expression plasmid. (5) With the help of T7 RNA polymerase, *E. coli* produce the target gene mRNA and target protein (blue/right). Notes: IPTG is the chemical isopropyl β -d-1-thiogalactopyranoside, which is similar in structure to a naturally occurring lactose metabolite. The promoter upstream of the T7 RNA polymerase gene is the *lacUV5* promoter, a mutated version of the natural lac operon promoter from *E. coli*. Illustration made with BioRender⁴. [\[Image description\]](#)

Controls

An empty pET vector can be used as a negative induction control, and a validated pET vector containing a gene/cDNA can be used as a positive induction control.

Image Descriptions

Figure 1 image description:

A diagram of an *E. coli* cell labeled "*E. coli* BL21 (DE3)." Various parts of the diagram are numbered to show the steps in the cell resulting in expression of the cloned gene. (1) shows the lac repressor protein bound to IPTG, not associated with any DNA. (2) shows the the *E. coli* chromosome with an inset showing the T7 RNA pol gene under the control of the *lacUV5* promoter. (3) shows mRNA and protein for T7 RNA pol being expressed from the gene. (4) shows the expression plasmid with T7 RNA pol bound. The inset shows the polymerase bound at the T7 promoter, which controls the expression of the target gene. (5) shows mRNA and protein for the target being expressed from the target gene. ↩

Thumbnail

"Laboratory bacterial culture.jpg" ↗ by Soledad Mirand-Rottmann is licensed under [CC BY-SA 4.0](#) ↗.

Image description: Bacterial cultures of transgenic *E. coli* grown in glass flasks overnight.

Author

Juliet Fuhrman, Erica Polleys, and Katherine Mattaini, Tufts University

-
1. Studier, F. W., and B. A. Moffatt. 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *Journal of Molecular Biology* 189:113–130. ↩
 2. (2011). *pET System Manual 11th Edition* (User Protocol TB055 Rev. C 0611JN). Novagen. ↩
 3. Gallego, Adriana. “19 Common Questions about Kanamycin.” *GoldBio*, goldbio.com/articles/article/19-Common-Questions-About-Kanamycin ↗. Accessed 23 Sept. 2024. ↩
 4. Created in BioRender. Mattaini, K. (2023) BioRender.com/n55c975 ↗. ↩
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25: QIBC

Summary

QIBC is an automated, high-throughput analysis method to collect and display data on large numbers of single cells from immunofluorescence microscopy images. The data is displayed in a scatter diagram and resembles data from a [flow cytometry](#) experiment.

Also known as:

Quantitative image-based cytometry

Samples needed

Immunofluorescence images

Method

A computer program is used to analyze [immunofluorescence](#) images of a sample. The program identifies the intensity of each color fluorescence used on a single-cell basis. Then each cell is plotted as a dot on a scatterplot. Therefore, the scatterplot represents the *population* of cells in each sample.

Controls

Not controls per se, but it is important in this analysis to make sure that images aren't saturated, that a large number of cells (5,000-10,000) are analyzed, as well as other quality control measures.

Interpretation

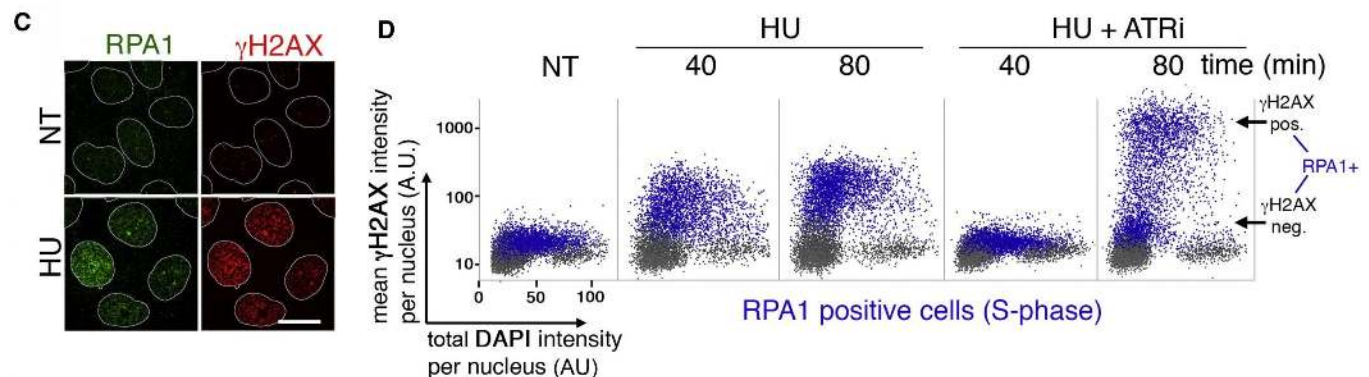


Figure 1. QIBC analysis of U-2-OS osteosarcoma cells treated with either hydroxyurea (HU) alone or HU with an ATR inhibitor (ATRi). Relevant section of caption for published figure reads: “(C) U-2-OS cells were treated with HU for 40 min, pre-extracted, and immunostained with the indicated antibodies. Scale bar, 10 μ m. (D) Quantitative image-based cytometry single-cell analysis (QIBC) of immunolabeled U-2-O-S cells. Asynchronous cells were treated with HU (2 mM) and ATRi (2 μ M) for the indicated times and immunostained as in (C). Total nuclear intensity for DAPI, mean nuclear intensities for RPA1 (chromatin-bound fraction [CB]), and γ -H2AX were determined for each of >5,000 individual cells and were plotted in a scatter diagram. S phase cells, with chromatin-loaded RPA1, are labeled in blue.” “Figure 1” by Toledo et al.^[1]. [\[Image description\]](#)

In Figure 1 above, panel C shows an example of the images on which QIBC was performed. Panel D shows the scatterplots made through QIBC analysis. In this diagram, blue dots represent cells that are positive for RPA1 that is chromatin-bound, or in other words, cells that are in S-phase. Grey dots are in other phases of the cell cycle. A dot placed farther right on the x-axis has a greater DAPI signal than one on the left. A dot placed farther up on the y-axis has a greater γ H2AX signal than one further down.

HU depletes dNTPs, the building blocks of DNA. This leads to replication stress and stalled replication forks. HU treatment increases the proportion of cells that are γ H2AX positive compared to cells with no treatment (NT). However, simultaneous

treatment with HU and an ATR inhibitor leads to more cells with very high amounts of γ H2AX, a hallmark of ATM activation.

Image Descriptions

Figure 1 image description:

Panel C shows four immunofluorescence microscopy images. In all images, nuclei are surrounded by a white line. No treatment images show no discernible nuclear RPA1 or γ H2AX expression. With HU treatment, there is significant nuclear RPA1 and γ H2AX expression.

Panel D shows the results of QIBC analysis in five scatterplots. Each plot contains approximately 5,000-10,000 data points. The x-axis measures total DAPI intensity per nucleus (A.U.), and the y-axis measures mean γ H2AX intensity per nucleus (A.U.). In each plot, grey data points are RPA1-negative. These show a range of DAPI intensities (10-100 A.U.) and low γ H2AX intensity (10-30 A.U.) in all cases. Blue data points show RPA1-positive cells and vary depending on conditions.

- No treatment: RPA1-positive cells are similar to RPA1-negative cells with low γ H2AX intensity (30-50 A.U.).
- HU, 40 min: Increasing levels of γ H2AX, 30-300 A.U.
- HU, 80 min: Mostly intermediate levels of γ H2AX, 90-300 A.U.
- HU + ATRi, 40 min: RPA1-positive cells are similar to RPA1-negative cells with low γ H2AX intensity (30-50 A.U.).
- HU + ATRi, 80 min: There are two main populations of RPA1-positive cells. The first population has relatively low γ H2AX intensity (30-50 A.U.). The second population has high γ H2AX intensity (800-1000 A.U.). The density of points between the two is sparse but appreciable.

↩

Thumbnail

"Figure 1. IR-induced γ H2AX foci number is dependent on the cell's DNA content" ↗ by Besse et al. is licensed under [CC BY-NC-ND 4.0](#) ↗. See citation 2.

Description: Results of a QIBC experiment.

Author

Katherine Mattaini, Tufts University

1. Toledo, L. I., M. Altmeyer, M. Rask, C. Lukas, D. H. Larsen, L. K. Povlsen, S. Bekker-Jensen, N. Mailand, J. Bartek, and J. Lukas. 2013. ATR prohibits replication catastrophe by preventing global exhaustion of RPA. *Cell* 155:1088-1103. ↩
 2. Besse, L., T. Rumiak, A. Reynaud-Angelin, C. Messaoudi, M.-N. Soler, S. A. E. Lambert, and V. Pennaneach. 2023. Protocol for automated multivariate quantitative-image-based cytometry analysis by fluorescence microscopy of asynchronous adherent cells. *STAR Protocols* 4:102446. ↩
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26: Quantitative Real Time PCR

Summary

Quantitative real time PCR (qRT-PCR) is used to determine the amount of a specific DNA sequence in a DNA mixture.

Also known as

qRT-PCR, qPCR, real time PCR

Caution

Note that the acronym RT-PCR can be confusing, as both real time and [reverse transcription PCR](#) can be abbreviated “RT,” so care should be taken to avoid confusion.

Samples needed

Sample of DNA, with a mixture of sequences

Method

qRT-PCR is a quantitative variant of typical [PCR](#). It is used to determine how much of a particular DNA sequence is present in a mixture. This can be used to determine gene expression levels (following [RT-PCR](#) of cellular RNAs), gene copy number, and for other applications.

With a pair of primers that amplify the target DNA, the amount of the target should double in each PCR cycle. The principle of qRT-PCR is that the less abundant the target DNA sequence in the sample, the more cycles it will take for the abundance of the target to reach a certain **threshold**. The number of cycles required to reach the threshold is known as the Ct value. Therefore, in two samples, the one with a lower Ct value indicates that the target DNA is more abundant in that sample.

In order to track the production of target DNA in real time, qRT-PCR uses a special machine that can detect fluorescence over the course of the PCR program. In addition to the usual PCR reagents, qRT-PCR requires a method for tracking production of the target. One method uses dyes that only fluoresce when they bind to dsDNA. This is the case for the popular reagent SYBR green. The second method uses probes that are specific to the target DNA sequence and which only fluoresce when they are cleaved by *Taq* DNA polymerase. These are called Taqman probes. Each of these methods has benefits and drawbacks. In both cases, a certain fluorescence signal is chosen as the threshold value.

qRT-PCR data is either compared to a standard curve or to an internal reference sample to determine absolute or relative DNA quantities, respectively.

Controls

As with any PCR protocol, primer specificity should be considered. If using intercalating dyes like SYBR green, a melt curve can be used to determine how many PCR products are produced in each reaction. After the normal PCR program runs, the temperature of the mixture is raised, and the change in fluorescence is monitored. When a product's melting temperature is reached, there is a peak in the ΔF signal. More than one peak in the melt curve indicates that the primers are not entirely specific.

When using qRT-PCR to measure gene expression, an endogenous control sequence is often amplified in addition to the sequence of interest. The endogenous control should be a gene whose expression is known to be very stable and is not affected by experimental treatments. This can be used to normalize the signal from the sequence of interest (to account for small differences in total cDNA added). Other controls relating to the [reverse transcription](#) step should also be used.

Furthermore, sometimes a no template control (water instead of DNA sample) is used to ensure that none of the other reagents are contaminated with DNA.

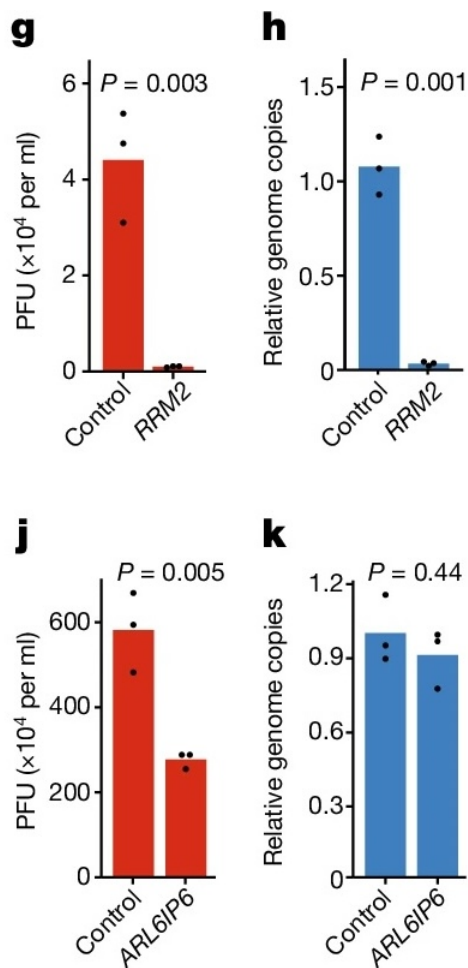


Figure 1. Virus production (left, red) and genomic copy number (right, blue) of human cytomegalovirus (HCMV) in the context of CRISPR-generated host gene knockouts. Relevant section of caption for published figure reads: “g,j, RRM2-knockout (g), ARL6IP6-knockout (j) and control cells were infected with HCMV-GFP virus. At 7 dpi viral supernatant was used to infect recipient wild-type fibroblasts. Forty-eight hours later, the percentage of GFP-positive recipient cells was determined by flow cytometry and used to calculate the number of plaque-forming units (PFU). n = 3 biological replicates. h,k. DNA was extracted from RRM2-knockout (h), ARL6IP6-knockout (k) and control cells infected with HCMV at 3 dpi and viral DNA was quantified using quantitative PCR (qPCR) and normalized to host DNA. n = 3 biological replicates.” “Figure 2” by Finkel et al.^[1]. [Image description]

In this experiment, researchers performed a CRISPR-generated knockout screen to ask what host cell genes are involved in human cytomegalovirus (HCMV) infection. Two knockouts are shown: *RRM2* in the top row & *ARL6IP6* in the bottom row. In panels g & j (left, red), the plaque-forming units (PFU) generated in each type of knockout cell are shown. PFU correlates with the number of viral particles that can infect new cells. Both knockouts significantly reduce the amount of functional viral particles formed. Panels h & k (right, blue) show the results of a qRT-PCR experiment to quantify the relative number of HCMV genome copies in each knockout cell compared to controls. While *RRM2* knockout almost completely abrogates the ability of HCMV to copy its genome, *ARL6IP6* does not significantly affect HCMV viral copy number. Therefore, *ARL6IP6* mainly acts on a step other than viral genome copying.

Image Descriptions

Figure 1 image description:

Four column graphs. Panels g and j measure PFU in various samples. Panel g shows $\sim 4.3 \times 10^4$ PFU per ml in control cells and almost 0 in RRM2 knockout cells with $p = 0.003$. Panel j shows $\sim 580 \times 10^4$ PFU per ml in control cells and $\sim 270 \times 10^4$ PFU per

ml in ARL6IP6 knockout cells with $p = 0.005$. Panels h and k show relative genome copies compared to control cells. Panel h shows nearly 0 genome copies in RRM2 knockout cells with $p = 0.001$, and panel k shows ~ 0.85 genome copies in ARL6IP6 knockout cells with $p = 0.044$. ↩

Thumbnail

"Qpcr-cycling.png" ↗ by Zuzanna K. Filutowska is licensed under [CC BY-SA 3.0](#) ↗.

Description: qPCR amplification plot.

Author

Katherine Mattaini, Tufts University

-
1. Finkel, Y., A. Nachshon, E. Aharon, T. Arazi, E. Simonovsky, M. Dobešová, Z. Saud, A. Gluck, T. Fisher, R. J. Stanton, M. Schwartz, and N. Stern-Ginossar. 2024. A virally encoded high-resolution screen of cytomegalovirus dependencies. *Nature* 630:712–719. ↩
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27: Reverse Transcription PCR

Summary

Reverse transcription PCR is a method that is used to synthesize a complementary DNA sequence from an RNA template.

Also known as

RT-PCR

Caution

Note that the acronym RT-PCR can be confusing, as [qRT-PCR](#) (a different method) is sometimes referred to as [real time PCR](#), so care should be taken to avoid confusion.

Samples needed

Sample of RNA, which can be a mixture of sequences

Method

The vast majority of the time, DNA serves as a template for RNA synthesis (in transcription), but not the reverse. However, the discovery of retroviruses illuminated the existence of reverse transcriptase (RT) enzymes, which use RNA as a template for DNA synthesis.

In molecular biology, RT-PCR is used to create cDNAs as an intermediate step in another experiment, or for molecular cloning. A cDNA is a “complementary” DNA, complementary to a mature mRNA. In eukaryotes, this means that a cDNA differs substantially from genomic DNA, since cDNA lacks introns.

To amplify a particular RNA sequence, two sequence-specific primers are used. However, RT-PCR is often used to create a cDNA pool of all cellular mRNAs, like in [qRT-PCR](#). In this case, primers with random sequences can be used, or polyT primers that anneal to polyA tails of mRNAs.

Generally, the protocol for RT-PCR is very similar to that of typical [PCR](#), with the exception that reverse transcriptase is used instead of DNA polymerase.

Controls

As with typical [PCR](#), primer specificity should be tested. Additionally, a no template control (water instead of RNA sample) is used to ensure that none of the other reagents are contaminated with template RNA. Lastly, a no reverse transcriptase control can be used to test the presence of gDNA contaminating the RNA sample.

Thumbnail

["Reverse transcriptase 1KLF.png"](#) ↗ by Thomas Splettstoesser is licensed under [CC BY-SA 3.0](#) ↗.

Description: Surface representation of the crystallographic structure of HIV reverse transcriptase based on [PDB coordinates 3KLF](#) ↗.

Author

Katherine Mattaini, Tufts University

27: Reverse Transcription PCR is shared under a [CC BY-SA 4.0](#) license and was authored, remixed, and/or curated by LibreTexts.

- [36: Western Blot](#) by [Katherine Mattaini](#) is licensed [CC BY-SA 4.0](#).

28: RNA Interference

Summary

RNA interference uses short RNA sequences complementary to a transcript of interest to decrease gene expression through transcriptional or translational silencing.

Also known as

RNAi, knockdown (different from knock-out); shRNA (“hairpins”) or siRNA

Samples needed

Cells to be treated

Method

siRNAs (small interfering RNAs) are short, ~19-21 basepair pieces of double-stranded RNA. They are processed by endogenous machinery in many cells, resulting in a temporary decreased expression of the target gene, usually on the scale of a few hours to a few days. siRNAs are transfected into cells, and once they degrade, expression of the gene of interest reverts to normal levels.

shRNAs (short hairpin RNAs) are longer than siRNAs and have a tight, hairpin turn on one end. When shRNAs are present in many cells, endogenous cell machinery processes them into siRNAs. shRNAs have the advantage of being delivered on a plasmid or viral vector. If the plasmid or vector contains a resistance gene, cells can be selected so that only those with genomic integration of the plasmid or vector remain. shRNAs can also be conditionally expressed, so that the gene of interest is only targeted upon administration of a certain compound, such as doxycycline. This allows for transient knock-down of genes whose complete knock-down would be lethal to the cells.

Controls

Usually [qPCR](#) or [western blot](#) are used to assess expression of gene of interest. Frequently, knockdown does not completely eliminate expression of the target gene. Furthermore, sh or siRNAs with different sequences will silence gene expression to different extents. Therefore, an analysis of the degree of silencing is a critical control and should always be shown.

Furthermore, in order to assess baseline levels of expression, a negative control si or shRNA should be used. Often, researchers will use an RNA that targets GFP, which is not normally present in cells outside of certain marine organisms, like the jellyfish from which GFP was first isolated. Sometimes an empty vector is used.

Lastly, when designing sh or siRNAs, researchers should always check that the target sequence does not appear in the genome anywhere besides the gene of interest. Otherwise, there can be unintended effects due to silencing of multiple genes.

Interpretation

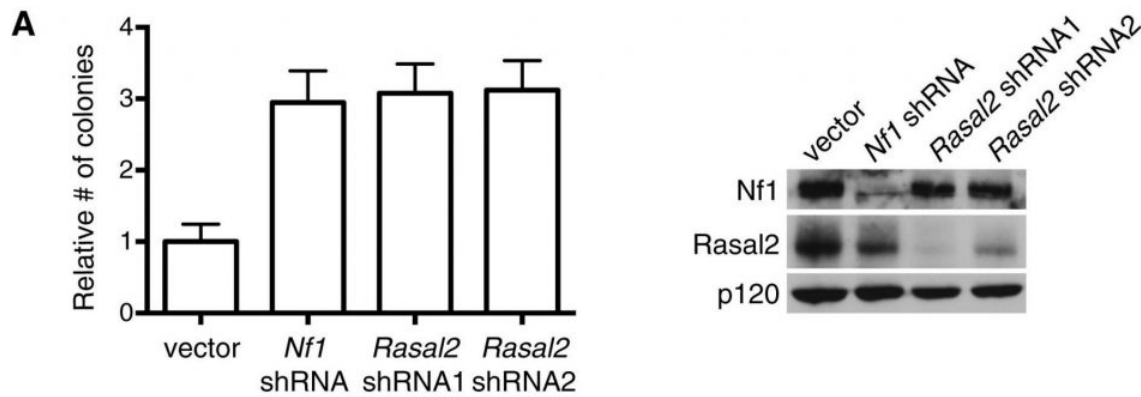


Figure 1. RNAi used to assess effect of loss of gene expression on colony formation in soft agar. Relevant section of caption for published figure reads: “(A) Left: immortalized MEFs were infected with lentiviral shRNAs that target *Rasal2*, *Nf1*, or control and were plated in soft agar. Data are reported as relative number of colonies $\pm$ SEM. Inactivation of *Nf1* or *Rasal2* induced a statistically significant increase in anchorage-independent growth ($p \leq 0.0001$). Right: western blot confirming knockdown.” “Figure 1” by McLaughlin et al. ^[1] [Image description]

In Figure 1 above, the authors show that loss of expression of either *Nf1* or *Rasal2* results in three times as many colonies in a soft agar colony formation assay. Importantly, they verify that *Nf1* and *Rasal2* expression is actually decreased by performing a Western blot (right). The reader can see that expression of the *Nf1* protein is decreased by the *Nf1* shRNA but not the *Rasal2* shRNAs or the empty vector control. *Rasal2* shRNAs only decrease *Rasal2* protein expression, and *Rasal2* shRNA1 is more effective than *Rasal2* shRNA2. (These two shRNAs are complementary to different parts of the *Rasal2* sequence.) Finally, the empty vector control has no discernible effect on *Nf1* or *Rasal2* protein levels.

Image Descriptions

Figure 1 image description:

A bar graph and Western blot. The bar graph shows relative number of colonies. *Nf1* shRNA, *Rasal2* shRNA1, and *Rasal2* shRNA2 all have three times as many colonies as vector control. The Western blot shows loss of *Nf1* expression only with *Nf1* shRNA. *Rasal2* shRNA1 shows nearly complete loss of *Rasal2* expression; *Rasal2* shRNA2 shows loss of most *Rasal2* expression. p120 loading control is consistent in all samples. ↩

Thumbnail

"201412 RNAi blue.png" ↗ by DataBase Center for Life Science (DBCLS) is licensed under CC BY 4.0 ↗.

Description: Schematic of mRNA being cleaved when bound to the RISC complex.

Author

Katherine Mattaini, Tufts University

1. McLaughlin, S. K., S. N. Olsen, B. Dake, T. De Raedt, E. Lim, R. T. Bronson, R. Beroukhi, K. Polyak, M. Brown, C. Kuperwasser, and K. Cichowski. 2013. The RasGAP gene, *RASAL2*, is a tumor and metastasis suppressor. *Cancer Cell* 24:365-378. ↩

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29: Sanger Sequencing

Summary

Sanger sequencing is a sequencing-by-synthesis method used to determine the nucleotide sequence of a short molecule of DNA. Sanger sequencing is appropriate when the length of the DNA molecule to be sequenced is 100-1000 nt.

Also known as:

Dideoxy sequencing

Chain termination method

Samples needed

To perform Sanger sequencing, you need:

1. DNA template
2. DNA oligonucleotide primer that anneals to the 3' end of the DNA template. In the manual version of Sanger sequencing, the primer is radioactively labeled.
3. Solution of dNTPs
4. Solutions of each of the 4 dideoxynucleotide triphosphates (ddNTPs). In automated Sanger sequencing, each of the 4 types of ddNTPs is labeled with a different colored fluorophore.
5. DNA polymerase
6. Polyacrylamide gel electrophoresis apparatus (for manual sequencing) or a machine that reads Sanger sequencing reaction products using capillary gel electrophoresis and a laser detector (for automated sequencing)

Method

Sanger sequencing is a specialized DNA synthesis reaction invented by Fred Sanger. The original method used radioactively labeled DNA molecules and polyacrylamide gel electrophoresis. In modern, automated Sanger sequencing, a double-stranded DNA molecule to be sequenced is incubated with a primer that anneals to its 3' terminus, dNTPs, fluorescently labeled ddNTPs (each type of ddNTP is labeled with a different fluorophore), and a DNA polymerase (Fig. 1). The concentration of the ddNTPs is much lower than the concentration of the dNTPs, so there is a small probability that a ddNTP will be incorporated opposite its complementary base at each position. Once the reaction is completed, the products consist of a collection of fragments, each labeled at its 3' end with a fluorophore corresponding to the ddNTP that terminated the synthesis reaction. These fragments are separated using capillary gel electrophoresis and the fluorophore present on each molecule is detected. A computer processes the data to create a [chromatogram](#) that shows the DNA sequence that was synthesized. The sequence of the original DNA template is the reverse complement.

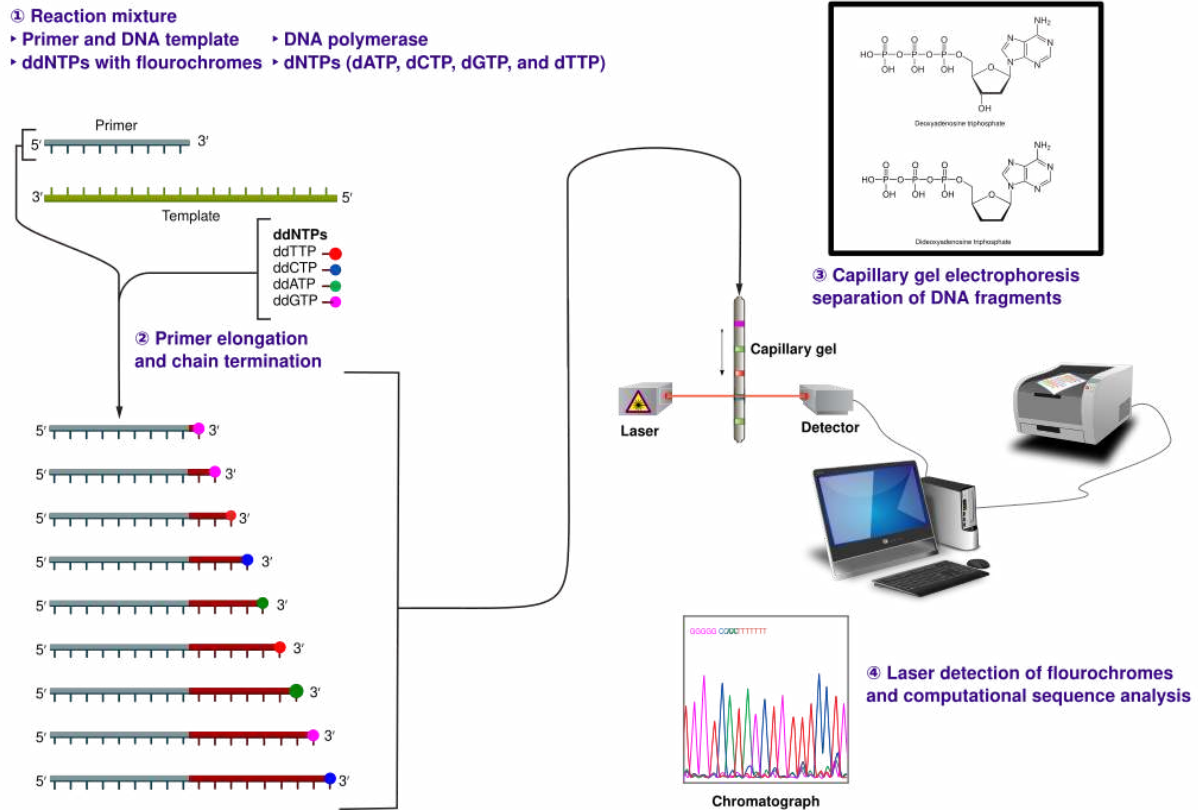


Figure 1. Sanger sequencing using fluorescently labeled ddNTPs. For details, see "Method." "Sanger-sequencing" by Estevez is licensed under CC BY-SA 3.0. [Image description]

Controls

Typically, no controls are used for Sanger sequencing. Sometimes, the DNA template is sequenced from both directions (using primers that anneal to each end of the double-strand template), and the sequence output is compared for consistency.

Interpretation

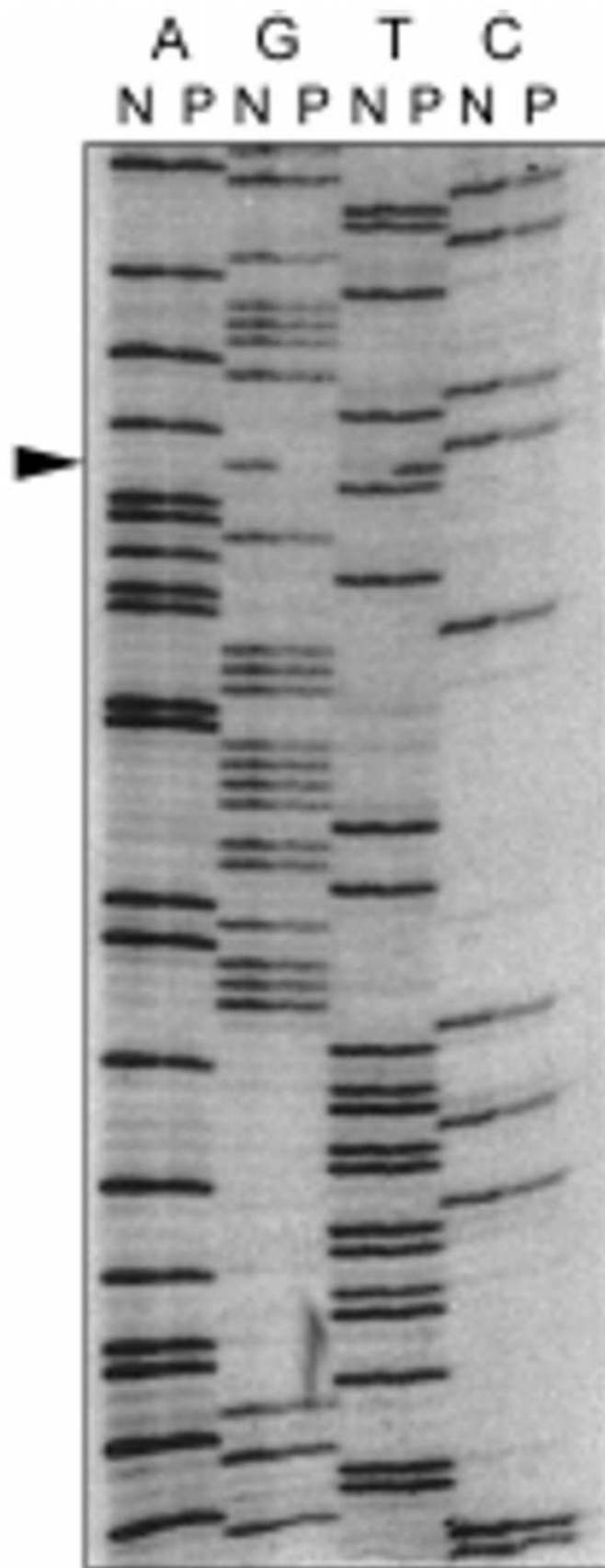


Figure 2. Manual sanger DNA sequencing of exon 17 in control (N) and MD-138 muscular dystrophy patient (P). The arrow indicates the position of the nucleotide substitution (G to T at position 35). "Figure 2" by Chaturvedi *et al.*^[1] [Image description]

This figure shows the results of Sanger DNA sequencing reactions using a dystrophin gene fragment as a template, a radioactively labeled primer, and ddATP, ddGTP, ddTTP, and ddCTP in four separate reactions, for two individuals side-by-side. The individual without muscular dystrophy has a G at position 35, while the individual with muscular dystrophy has a T at this position. The sequence is read from the bottom of the gel to the top of the gel, with the smaller fragments corresponding to synthesis reactions that terminated early near the 3' end of the template.

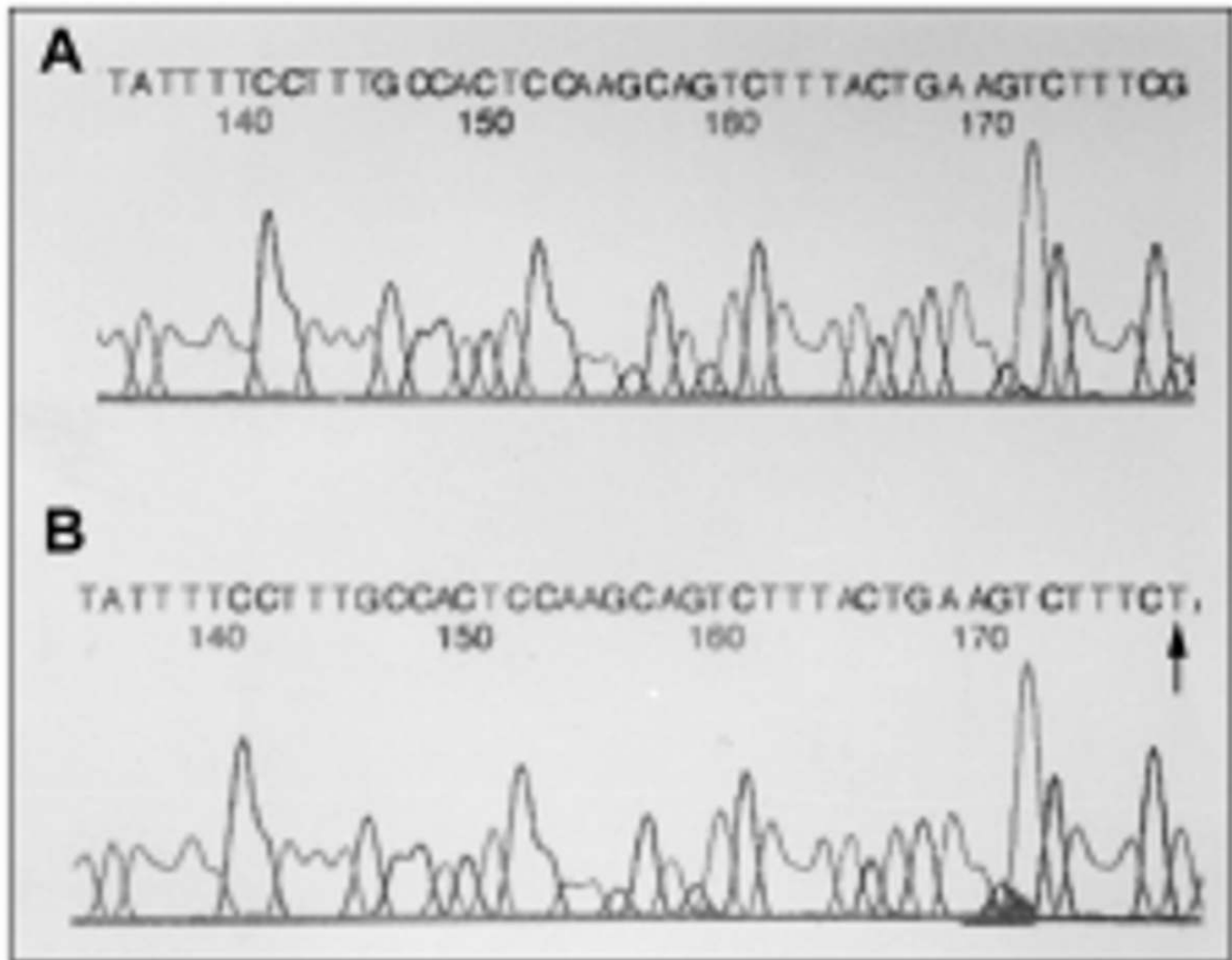


Figure 3. Automated DNA sequencing of exon 17 of normal male control and members of family, MD-28. The arrow indicates the nucleotide substitution C2278T in the exonic region of the patient, the heterozygous condition in the mother, and normal sequence in the father and unaffected brother. Figure 3 by Chaturvedi *et al.*^[1] [Image description]

This figure shows the [chromatograms](#) for four family members, including an individual with muscular dystrophy. The patient has a peak corresponding to a synthesis fragment that terminated with a T, while their brother and father have peaks corresponding to C (which is the same as the control sequence). The mother is heterozygous for the T and C peaks. The patient inherited the chromosome with the T peak from his mother.

Image descriptions

Figure 1 image description:

A diagram of automated Sanger sequencing using fluorescently labeled ddNTPs. ↩

Figure 2 image description:

The results of a sequencing gel used to separate DNA synthesis products of the dystrophin gene, created using radioactively labeled primers in separate Sanger sequencing reactions with four different ddNTPs. There is a single difference in the sequences between the two individuals, with the muscular dystrophy patient having a T where the arrow is pointing. ↩

Figure 3 image description:

The results of five automated Sanger sequencing reactions giving portions of the dystrophin gene sequence for a control sample and four family members. The base calls are represented by peaks at each position within the gene. The sequence corresponding to the peaks is written below each line, with the corresponding positions indicated by numbers. The differences in the sequences are indicated by arrows. ↩

Thumbnail

"Skraban–Deardorff syndrome — Sanger sequencing (cropped)" ↗ by Jiacheng Hu; Mingming Xu; Xiaobo Zhu; Yu Zhang is licensed under CC BY 4.0 ↗.

Author

Mitch McVey, Tufts University

1. Chaturvedi, L. S., M. Mukherjee, S. Srivastava, E. D. Mittal, and B. Mittal. 2001. Point mutation and polymorphism in Duchenne/Becker Muscular Dystrophy (D/BMD) patients. *Experimental and Molecular Medicine* 33: 251-256. [Return to Figure 2.](#) [Return to Figure 3.](#)

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30: Site-directed Mutagenesis

Summary

This method uses [PCR](#) to edit specific nucleotides in a DNA sequence. Most often this technique is used to study the importance or function of certain sequences and their resulting proteins.

Also known as

Site specific mutagenesis or oligonucleotide-directed mutagenesis

Samples needed

To perform this method you first need to determine what sequence you want to edit and design forward and reverse primers that correspond to your intended mutations. In addition to primers, this process requires plasmid DNA with the sequence of interest which will be edited via PCR

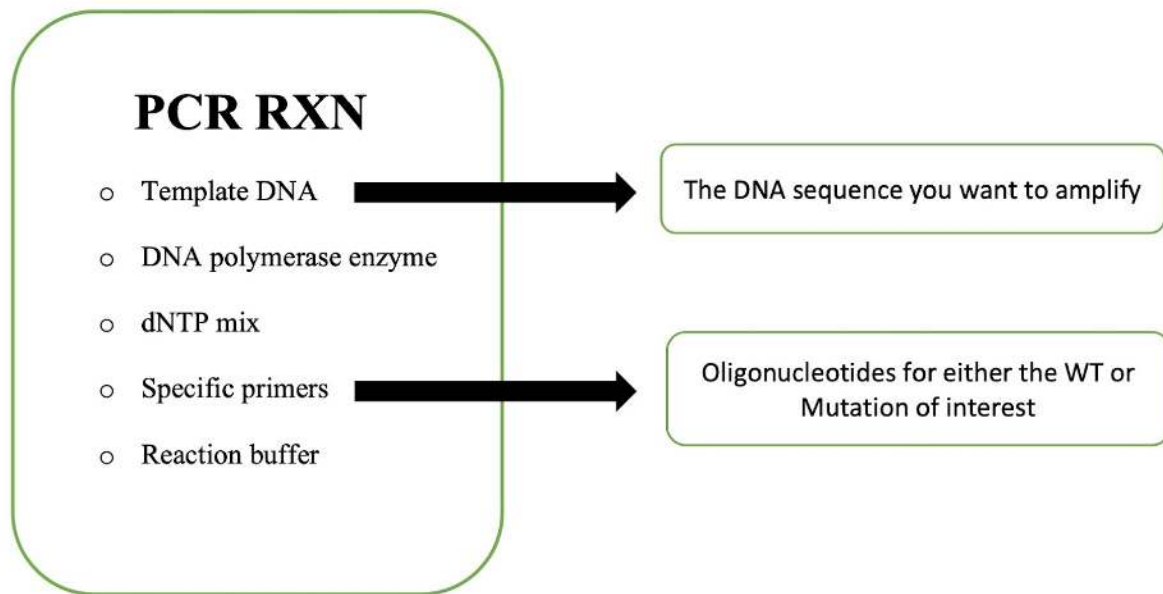
Method

The first step in this experimental workflow is to perform PCR amplification using your primer pairs of interest combined with template plasmid DNA. The results of this amplification will be many copies of the new mutated DNA sequence as well as the few remaining copies of your original template circular DNA plasmid.

These new mutated products will be linear and must be circularized before transformation into bacterial cells. This process can be accomplished a number of different ways, one of which being treatment with a cocktail of enzymes (KLD mix): kinase, ligase, and *dpnI*, used to prepare the sample. Within this mixture, kinase phosphorylates the 5' end of each of your linear strands which allows the ligase to join the two ends together resulting in a circular product.

The last step in this process is degrading the remaining template DNA to increase the number of mutants recovered in future steps. To accomplish this we must have a way to degrade only the template plasmids without damaging the newly amplified PCR products. Luckily, *E. coli* cells contain specialized enzymes which work to methylate GATC sites as a way to recognize parental vs replicated DNA strands. Knowing this, we can use *dpnI*, an enzyme which specifically works to cut methylated GATC sites, to clear the unwanted sequences. These products are now ready to be transformed into bacterial cells so the mutation's function can be assessed.

Step 1. Amplification



Step 2. Re-circularizing

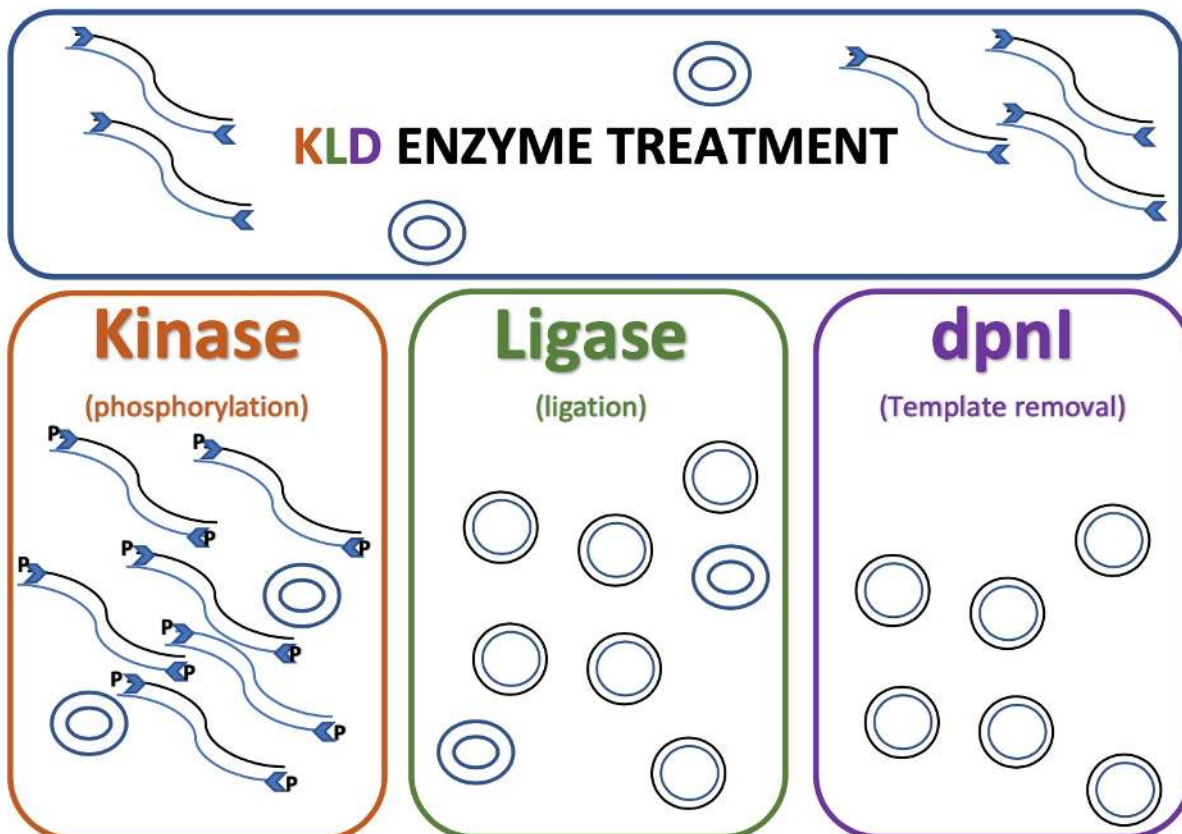


Figure 1. Overview of the method of site-directed mutagenesis. [\[Image description\]](#)

Controls

It is important to use wild type (WT) primers in your experiment as a control to assess how mutations in the plasmid sequence affect downstream form and function.

Interpretation



Figure 2. Real world uses for site-directed mutagenesis in the news. A common use for this experimental technique is enzyme editing. Because enzymes act as catalysts for chemical reactions, their efficiency and specificity are important in determining the rate of biological reactions. Site-directed mutagenesis has long been used to optimize these enzymes' performances by making them more specific for certain reactions or improving their thermostability. Citations at end of entry. [\[Image description\]](#)

Although results of this method are not typically seen until the new plasmids are transformed into bacterial cells (where they will undergo large-scale, *in vivo* replication), the success of the process could be visualized using Sanger sequencing. This type of DNA sequencing allows researchers to identify changes in small sections of the genome, perfect for identifying mutations such as insertions, deletions or single nucleotide changes.

Image descriptions

Figure 1 image description:

A schematic of the method of site-directed mutagenesis.

Step 1: Amplification. PCR Reaction mix includes template DNA (the sequence you intend to amplify), DNA polymerase, dNTP mix, specific primers (oligonucleotides for either WT or mutation of interest), and reaction buffer.

Step 2: Re-circularizing. This involves treatment with a kinase to phosphorylate the 5' ends of the DNA, a ligase to join the linear PCR products into a circle, and DpnI, to digest the template DNA. [↩](#)

Figure 2 image description:

Images shows headlines from studied utilizing site-directed mutagenesis. These studies can be found in the citations below. The text states: "Site-directed mutagenesis in the news. This method has been popular in optimizing enzyme performance and specificity." [↩](#)

Thumbnail

"[Site-Directed-Mutagenesis.gif](#)" ↗ by Stephengregg is licensed under [CC BY-SA 3.0\(opens in new window\)](#) ↗.

Description: An animation showing the basics of site directed mutagenesis.

Author

Emilie Jones, Tufts University

-
1. Chen, Z., J. Chen, W. Zhang, T. Zhang, C. Guang, and W. Mu. 2018. Improving Thermostability and Catalytic Behavior of l-Rhamnose Isomerase from *Caldicellulosiruptor obsidiansis* OB47 toward d-Allulose by Site-Directed Mutagenesis. *Journal of Agricultural and Food Chemistry*.
 2. Meng, T., S. Bezstarosti, U. Singh, M. Yap, L. Scott, N. Petrosyan, F. Quiroz, N. V. Eps, E. K.-W. Hui, D. Suh, Q. Zhu, R. Pei, C. S. M. Kramer, F. H. J. Claas, D. Lowe, and S. Heidt. (n.d.). Site-directed mutagenesis of HLA molecules reveals the functional epitope of a human HLA-A1/A36-specific monoclonal antibody.
 3. Yamamoto, T., S. Fujiwara, Y. Tachibana, M. Takagi, K. Fukui, and T. Imanaka. 2000. Alteration of product specificity of cyclodextrin glucanotransferase from *Thermococcus* sp. B1001 by site-directed mutagenesis. *Journal of Bioscience and Bioengineering* 89:206–209.
-

30: [Site-directed Mutagenesis](#) is shared under a [CC BY-SA 4.0](#) license and was authored, remixed, and/or curated by LibreTexts.

31: Site-specific Recombination

Summary

Both the Cre-lox and FLP-FRT systems are used for site-specific recombination to facilitate genome editing, especially for achieving tissue or temporal specificity.

Also known as

Variants: Cre-lox, FLP-FRT

Samples needed

Cells or organisms to be modified

Method

Site-specific combination requires two elements: A recombinase enzyme that catalyzes DNA cleavage and re-ligation, and two recombination sites in the DNA. The two most commonly used recombinase-recombination site pairs are Cre-lox and FLP-FRT. The recombination sites are made up of an asymmetrical core region where the cuts occur, flanked by inverted repeats where the recombinase binds the DNA. For recombination to occur between two recombination sites, the core sequences must be identical and they must be aligned in the same direction in space, which can require looping of the DNA. The directionality of the recombination sites is determined by the asymmetrical core sequence.

If the two recombination sites are aligned in the same direction in a piece of DNA, when recombination occurs, the intervening sequence will be removed. If the two sites are pointing toward one another, the intervening sequence will be inverted.

Site-specific recombination is often used in animal models. One of the benefits of site-specific recombination is that the genomic modification can be confined to a specific tissue by using a recombinase (Cre or FLP) under the control of a tissue-specific promoter. Alternatively, temporal specificity can be achieved through the use of inducible recombinases, like the Cre-ER fusion protein. Cre-ER only translocates to the nucleus, and therefore can only catalyze site-specific recombination, in the presence of either tamoxifen or 4-OHT, which can be supplied orally or injected. So, in this case, the researchers control the point at which the site-specific recombination occurs.

Controls

Unmodified control

Interpretation

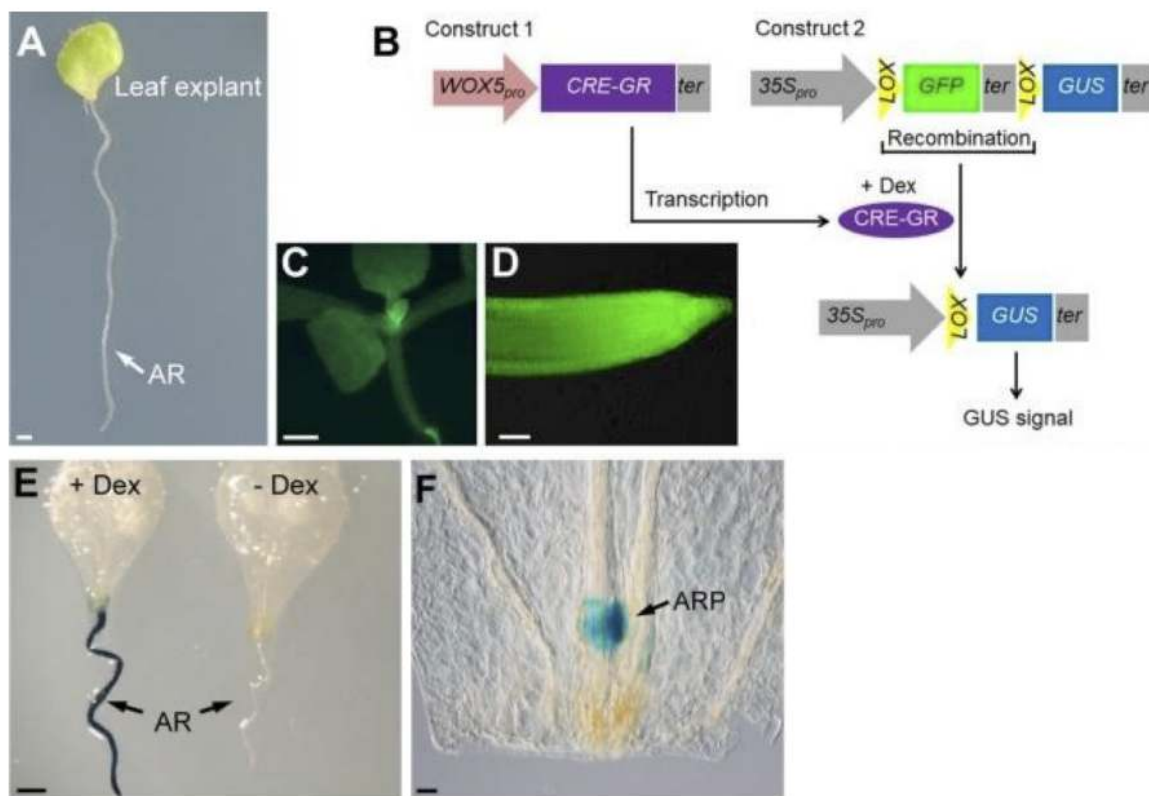


Figure 1. A Cre-lox system used for cell lineage tracing in *Arabidopsis*. Relevant section of caption for published figure reads: “CRE/LOX analysis of cell lineage in root formation. **A** Adventitious root formation from detached *Arabidopsis* leaves cultured on B5 medium for 10 d. **B** Schematic of analyses using CRE/LOX system under the control of *WOX5pro* to trace cell lineage in (C–L). Two independent lines were analyzed and showed the same results. Representative results are shown in (C–L). **C**, **D** GFP signals in seedling (**C**) and root (**D**) of F1 population. **E**, **F** GUS staining in *WOX5pro*-driven CRE/LOX analysis, showing positive GUS signal in the adventitious root (**E**) and adventitious root primordium (**F**) from detached leaves.” “Figure 1” by Zhai and Xu.
[\[1\] \[Image description\]](#)

In this experiment, researchers set out to identify all cells descended from cells expressing *WOX5*, which is known to be expressed in the root primordia & root stem cell niche. To do this, they used an inducible Cre-lox system. As shown in “Construct 2” in panel B, this system used two reporters. Before recombination, GFP is expressed, and after recombination, GUS is expressed. GFP is fluorescent, and GUS is an enzyme that can cleave a chemical to produce a blue precipitate. This construct is under the control of the 35S ribosomal RNA promoter, which is active in all cells.

In this system, the researchers used a Cre fusion protein, Cre-GR (glucocorticoid receptor). This protein is usually found in the cytosol and will only enter the nucleus in the presence of the chemical dexamethasone (dex). Therefore, in order for site-specific recombination to occur, the Cre-GR protein must be expressed (only occurs in tissues where the *WOX5* promoter is active) **and** dexamethasone must be provided by the researcher. Thus, the system provides both tissue specificity (*WOX5* promoter) and temporal specificity (dex administration). Once recombination occurs, the cell’s genome is permanently modified, and any *descendents* of that cell will also express GUS instead of GFP. In this way, one can identify all the descendants of particular cells. This kind of lineage tracing analysis is often used to identify all the descendants of particular stem cells.

In this experiment, the Zhai and Xu wanted to determine if the “adventitious” root cells, i.e. root cells that form from non-root tissue, like explanted leaves, are descendants of *WOX5*-expressing cells. In panels E and F, we can see that the adventitious root (AR) cells and adventitious root primordium (ARP) both express GUS in the presence of dex, indicating that they do indeed descend from *WOX5*-expressing cells. If their parent cell did *not* express *WOX5*, they would not express Cre-GR, recombination would not have occurred, and the descendants would all express GFP instead of GUS.

Image Descriptions

Figure 1 image description:

A: Photo of a leaf with an adventitious root (AR) growing from it.

B: Schematic of Cre-lox system. Construct 1: *WOX5pro* - Cre-GR - *ter*. Transcription leads to Cre-GR protein. Construct 2: *S35pro* - *LOX* - *GFP* - *ter* - *LOX* - *GUS* - *ter*. Cre-GR + dex leads to recombination to: *S35pro* - *GUS* - *ter*, which leads to GUS signal.

C: Photo of seedling showing green fluorescence.

D: Photo of root showing green fluorescence.

E: Photo of two explanted leaves with ARs growing from them. Left, +dex, shows dark blue staining throughout AR, whereas right, -dex, shows no staining.

F: Photo zooming in on bottom of explanted leaf. There is a spot of GUS staining near the bottom of the leaf labeled ARP for adventitious root primordium.

↩

Thumbnail

"Cre-lox inversion.svg" ↗ by Staticd is licensed under [CC BY-SA 3.0](#) ↗.

Description: Inversion of a DNA sequence between two inverted Lox sites.

Author

Katherine Mattaini, Tufts University

-
1. Zhai, N., and L. Xu. 2020. CRE/LOX-based analysis of cell lineage during root formation and regeneration in Arabidopsis. *aBIOTECH* 1:153–156. ↩
-

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- [36: Western Blot](#) by [Katherine Mattaini](#) is licensed [CC BY-SA 4.0](#).

32: Soft Agar Colony Formation Assay

Summary

The soft agar colony formation assay is a test to determine whether cells are anchorage-independent. Anchorage independence is a characteristic of cells that have undergone malignant transformation.

Also known as

Test of anchorage independence

Samples needed

Cells capable of growing in tissue culture

Method

Normal, non-transformed cells only grow attached to a solid substrate. This characteristic is called anchorage dependence. A distinctive feature of transformed cells is their ability to grow without that solid substrate; they are anchorage independent. To perform the test for anchorage independence, cells are plated in agar, a soft, jelly-like substance, and supplied with the necessary nutrients and growth factors. After 2-3 weeks, the plates are stained to aid in colony identification, then photographed, and the colonies are counted.^[1]

Controls

The soft agar colony formation assay is nearly always used to compare anchorage-independence of cells +/- a treatment or intervention. Therefore, the usual mock treatment controls apply.

Interpretation

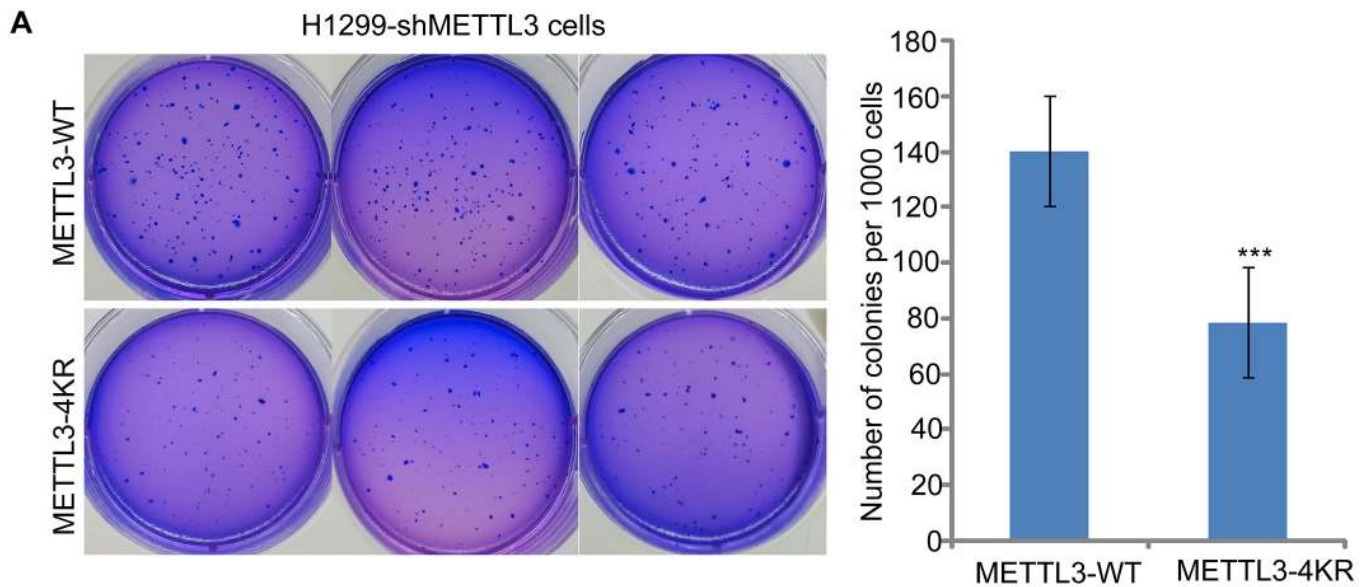


Figure 1. A soft agar colony formation assay on H1299 cells with either wild type METTL3 protein or METTL3 with a mutation that turns four key lysine residues to arginine residues to prevent SUMOylation. Relevant section of caption for published figure reads: “(A) The mutation of 4KR in METTL3 reduces the soft-agar colony formation of H1299 cells. H1299-shMETTL3 cells stably re-expressed METTL3-WT or METTL3-4KR cell lines were seeded in 2 ml of medium containing 10% FBS with 0.35% soft agarose at 1000 per well and layered onto 0.6% solidified agarose. The photographs were taken 14 days after seeding, and the number of colonies were counted and analysed. Each value represents the mean±s.e.m. of three independent experiments with triplicates.” “Figure 5” by Yuzhang Du, Guofang Hou, Hailong Zhang, Jinzhuo Dou, Jianfeng He, Yanming Guo, Lian Li, Ran Chen, Yanli Wang, Rong Deng, Jian Huang, Bin Jiang, Ming Xu, Jinke Cheng, Guo-Qiang Chen, Xian Zhao, Jianxiu Yu^[2]. [\[Image description\]](#)

This figure shows the reader that in H1299 non-small cell lung cancer(NSCLC) cells, the SUMOylation of METTL3 is important for the process of transformation. This is evident because if METTL3 cannot be SUMOylated (4KR mutant), significantly fewer colonies are able to form in soft agar.

Image Descriptions

Figure 1 image description:

Left: Six cell culture plates containing purple-stained soft agar. In each plate, there are some darker purple spots showing cell colonies. There are more colonies in the three wild type plates compared to the mutant plates. Right: A column graph quantifying colony growth. The wild type condition shows about 140 colonies per 1000 cells, whereas the mutant condition shows about 80. The difference is significant. ↩

Thumbnail

"Figure 2" ↗ by Wang et al. is licensed under [CC BY-NC 3.0](#) ↗. See reference 3.

Description: Colony formation assay plates.

Author

Katherine Mattaini, Tufts University

1. Borowicz, S., M. Van Scoyk, S. Avasarala, K. Karuppusamy Rathinam Manoj, J. Tauler, K. Bikkavilli Rama, and R. A. Winn. 2014. The soft agar colony formation assay. *JoVE* :e51998. ↩
2. Du, Y., G. Hou, H. Zhang, J. Dou, J. He, Y. Guo, L. Li, R. Chen, Y. Wang, R. Deng, J. Huang, B. Jiang, M. Xu, J. Cheng, G. Chen, X. Zhao, and J. Yu. 2018. SUMOylation of the m6A-RNA methyltransferase METTL3 modulates its function. *Nucleic Acids Research* 46:5195-5208. ↩
3. Wang, H., Z. Shao, Z. Xu, B. Ye, M. Li, Q. Zheng, X. Ma, and P. Shi. 2022. Antiproliferative and apoptotic activity of gemcitabine-lauric acid conjugate on human bladder cancer cells. *Iranian Journal of Basic Medical Sciences* 25:536–542. ↩

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33: TUNEL Assay

Summary

The TUNEL assay labels cells with extensive DNA fragmentation, often used to mark cells undergoing apoptosis.

Also known as

Terminal deoxynucleotide transferase (TdT) dUTP nick end labeling

Samples needed

Fixed cells or tissue in the format needed for final visualization

Method

TUNEL uses the enzyme terminal deoxynucleotide transferase (TdT) to add labeled dUTP to the 3' OH of DNA breaks. The label on dUTP depends on the desired visualization method. For instance, for a fluorescence microscopy application, dUTP can be directly attached to a fluorescent molecule. The more DNA breaks there are, the stronger the TUNEL signal will be. TUNEL is used to detect cells with massive amount of DNA damage, especially apoptotic cells, as DNA fragmentation is one step of the apoptotic death program.

To perform the TUNEL assay, samples are first fixed, then solubilized. Solubilizing creates holes in the plasma membrane to allow the reagents to enter the sample. The sample is treated with TdT and labeled dUTP, and visualized appropriately.

If a cell is positive, it has lots of DNA fragmentation.

Controls

Often times, controls for TUNEL staining are not shown in a published work. However, a positive control can be generated by treating a sample with DNase to cause DNA breakage before staining. A negative control can be performed if a critical assay reagent (i.e. TdT) is left out.

Thumbnail

"[Bmp7 functions to promote cell survival in the cloacal endoderm.jpg](#)" ↗ by Xu et al. is licensed under [CC BY 4.0](#) ↗.

Description: Fluorescence image of a TUNEL assay.

Author

Katherine Mattaini, Tufts University

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34: Transwell Migration and Invasion Assay

Summary

Transwell migration and invasion assays are used to determine if cells can move through a porous membrane (migration assay) or both the membrane and an extracellular matrix (invasion assay). Invasiveness is a characteristic feature of more advanced transformed cells, as it is necessary for metastasis.

Also known as

Boyden chamber assay, modified Boyden chamber assay

Samples needed

A plate of confluent cells in a monolayer

Method

A chemoattractant is placed in the lower chamber of a cell culture plate. The cells are placed on top of a porous transwell membrane alone for the mobility assay or one covered with extracellular matrix for the invasion assay. Following an incubation period that is specific to the cell type, cells that have crossed the membrane will be stained and counted^[1].

Controls

The transwell migration and invasion assays are nearly always used to compare mobility and/or invasiveness of cells +/- a treatment, intervention, or genetic difference. Therefore, the usual mock treatment controls apply.

Interpretation

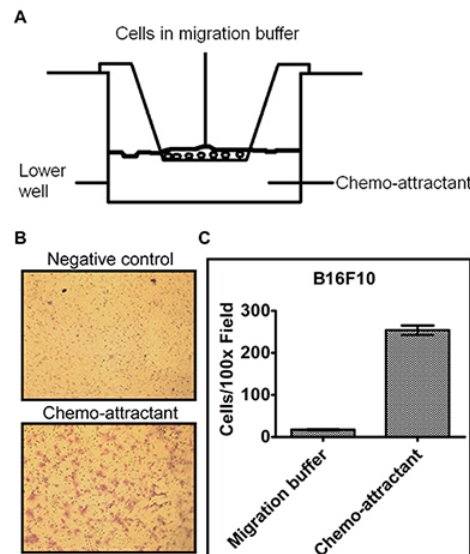


Figure 1. A schematic of the setup for a transwell assay and an example of representative results. Relevant section of caption for published figure reads: “(A) A diagram of the transwell insert apparatus used to measure cell migration and invasion. (B) Representative pictures of B16F10 cell transwell migration. Cell migration buffer and NIH3T3 cell conditioned medium were added to the lower chamber as the negative control and chemo-attractant, respectively. After cell migration and staining with crystal violet as described in the Procedure, pictures of the migrated cells (purple stained) were taken using a microscope with a 10x objective (total magnification 100x). Pores of the membranes could also be observed as the numerous small, round and dark colored dots in the picture. (C) Quantification of cells migrating toward the migration buffer or chemo-attractant (Average of 5 picture fields at 100x total magnification).” “Figure 3”(opens in new window) by Justus et al.^[1] [\[Image description\]](#)

This figure shows that B16F10 melanoma cells only cross the transwell membrane when the liquid in the lower chamber is cell medium that has been “conditioned” by NIH3T3 cells. (NIH3T3 are immortalized mouse embryonic fibroblasts.) This means that in a different cell culture dish, NIH3T3 cells were grown in cell culture medium, then the medium only (not the cells) were placed

in the lower chamber for the transwell assay. This “conditioned” medium will now contain any proteins and chemicals secreted by the NIH3T3 cells. The results show that the NIH3T3 conditioned medium contains a chemoattractant that promotes migration of the melanoma cells across the transwell membrane. Note that because there is no extracellular matrix used to coat the transwell insert, this is considered a migration assay and not an invasion assay.

Image Descriptions

Figure 1 image description:

Schematic and example data from a transwell migration assay. Panel A shows the schematic. The lower chamber is about halfway full of liquid containing a chemoattractant. The upper chamber rests on top, with the bottom of the upper chamber submerged in the liquid in the lower chamber. The cells are in the upper chamber. Panel B shows two images of cell stains. The image with chemoattractant shows more cells than that without. Panel C is a column graph showing between 200-300 cells per 100x field with chemoattractant and almost none without. ↩

Thumbnail

"EMT and vimentin in confined spaces.jpg" ↗ by Leggett et al. is licensed under [CC BY 4.0](#) ↗.

Description: Transwell (Boyden Chamber) assay for migration through confined pores.

Author

Katherine Mattaini, Tufts University

1. Justus, C. R., N. Leffler, M. Ruiz-Echevarria, and L. V. Yang. 2014. In vitro cell migration and invasion assays. JoVE :e51046. [\[Return to Method section\]](#) [\[Return to Figure 1\]](#)
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35: Ultracentrifugation

Summary

Ultracentrifugation is used to separate and purify macromolecules, including nucleic acids, according to their respective densities. While the technique requires expensive equipment and is time-consuming, it is very sensitive to small changes in physical properties.

Also known as

High-speed centrifugation

Isopycnic centrifugation

Density gradient centrifugation

Samples needed

To conduct ultracentrifugation, you need:

1. A mixture of DNA of different densities
2. A centrifuge tube containing a cesium chloride salt gradient
3. An ultracentrifuge

Method

To create the gradient, a mixture of DNA (or other macromolecule) of different densities is mixed with a cesium chloride (CsCl) solution and placed into a centrifuge tube. Upon centrifugation at extremely high speeds ($>200,000 \times g$) in an ultracentrifuge, CsCl spontaneously dissociates into Cs^+ and Cl^- ions, and the heavier Cs^+ ions move to the bottom of the tube. This movement is somewhat balanced by diffusion; therefore, the Cs^+ ions form a continuous gradient with higher density at the bottom of the tube.

When a DNA mixture is placed in the tube and the tube is subjected to extremely high centrifugal forces, the DNA molecules will separate in the gradient according to their relative densities, with more dense DNA layering at the bottom of the tube. The tube can then be punctured and pure DNA of a specific density can be purified by extracting it with a needle/syringe. Ethidium bromide or another intercalating agent is often included during ultracentrifugation to enable visualization of the DNA bands under ultraviolet light.

Controls

While controls are not usually included in this separation/purification scheme, DNA markers of known density can be included for comparison purposes.

Interpretation

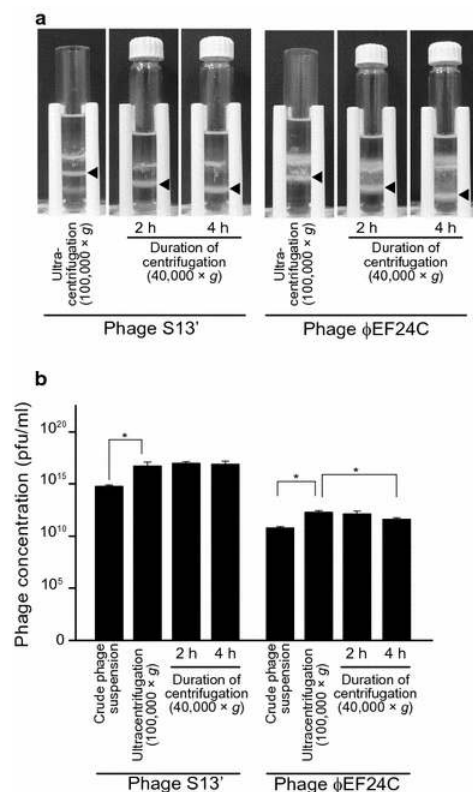


Figure 1. Virus purification by CsCl density gradient centrifugation. Relevant section of caption for published figure reads: “The crude suspensions of phages S13’ and φEF24C were purified by CsCl density gradient ultracentrifugation (100,000 × g, 1 h, 4 °C) and general centrifugation (40,000 × g, 2 h and 4 h, 4 °C). (a) Observation of virus bands. Black arrowheads indicate the phage bands. (b) Concentrations of phage bands. The phage band was collected from each tube, and phage concentration was measured. Bars indicate mean values with standard deviations. Statistical significance is indicated by asterisks ($P < 0.05$; $n = 6$).” “Figure 1” by Nasukawa *et al.*^[1] [Image description]

In part A of the figure, the bands corresponding to the S13’ and φEF24C phage strains following ultracentrifugation or general (lower-speed) centrifugation are shown in their centrifugation tubes (note: in this case, the bands likely contain both viral DNA and protein). The phage bands are indicated with black arrowheads and are distinctly separated from other nucleic acid bands in the mixture.

In part B of the figure, the number of phage particles was measured by calculating the number of plaque forming units (pfu) per milliliter of solution. Purification of the phage genomes by ultracentrifugation results in a concentration of phage about 1000-fold greater than that in a crude phage suspension, and equal to or greater than that obtained by general centrifugation at lower speeds. From these results, the authors concluded that regular centrifugation can be an effective method for purification of phage particles.

It should be noted that the duration of ultracentrifugation in these experiments (1 hour) was shorter than that of regular centrifugation. Typically, ultracentrifugation used to obtain extremely pure samples of specific nucleic acids is a long process, taking 12-24 hours.

Image Descriptions

Figure 1 image description:

Part A is a photograph of a set of centrifugation tubes containing phage DNA that has been subjected to centrifugation at both high and low speeds. The banding patterns in the tubes indicates that relatively pure populations of macromolecules have segregated by density to different positions in the tubes. Part B is bar graph showing the quantification of the number of phage in each of the bands. ↩

Thumbnail

"Beckman Model E Ultracentrifuge Rotor" ↗ by Beckman Instruments, Inc is in the [Public Domain](#) ↗.

Author

Mitch McVey, Tufts University

1. Nasukawa T., Uchiyama J., Taharaguchi S., Ota S., Ujihara T., Matsuzaki S., Murakami H., Mizukami K., and M Sakaguchi. Virus purification by CsCl density gradient using general centrifugation. 2017. Archives of Virology. **162**: 3523–3528. [↩](#)

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36: Western Blot

Summary

Western blots are used to determine relatively how much of a particular protein is present across at least two samples.

Also known as

Western, immunoblot

Caution

Nomenclature note: The [Southern blot](#) is named after its inventor, Edward Southern, and therefore, the word “Southern” is a proper noun and should always be capitalized in its name. However, the [northern blot](#) and western blot were named in reference to the Southern blot and the cardinal directions. Therefore, the words “northern” and “western” in the method names are not proper nouns, and should *not* be capitalized.

Samples needed

Mixtures or pure samples of proteins separated by SDS-PAGE

Method

The first step in performing a western blot experiment is to separate proteins based on their molecular weight via [SDS-PAGE](#). Next, the proteins are transferred from the gel to a membrane using an electric current. At this point, if desired, the researcher can reversibly stain the membrane to show total protein, with a stain like Ponceau S. After destaining, or immediately after transfer if staining is not performed, the membrane is blocked with a protein mixture to discourage non-specific antibody binding. Often this protein mixture is either milk or bovine serum albumin (BSA). Then, the membrane is incubated in an antibody that specifically binds the protein of interest. This is called the primary antibody. Usually, a secondary antibody is used for visualization. The secondary antibody binds the primary antibody. The secondary antibody is also conjugated to either a fluorophore, an enzyme catalyzes a colorimetric or luminescent reaction, or some other means of visualization. The result will be the presence of “bands,” i.e. signal, at the size of the protein of interest. The strength of the signal, i.e. the thickness of the band, semi-quantitatively measures the amount of the protein of interest present in the sample.

Controls

Molecular weight markers made of proteins of known size should be run alongside unknown samples to relate distance traveled in the gel to molecular weight of bands. Furthermore, measures should be taken to ensure that the same total amount of protein is loaded in each lane. This can be done either by staining the membrane for total protein before immunoblotting (i.e. with Ponceau or a similar stain), or by cutting the membrane horizontally and blotting the appropriate piece for a “housekeeping” protein like GAPDH, actin, tubulin, etc. In the latter method, the housekeeping protein is referred to as the loading control. Lastly, any antibody used should be tested for specificity using positive and negative controls for the protein of interest.

Caution

Sometimes Western blots are used to test for the presence of a specific post-translational modification, like a particular phosphorylation site. Often, phosphorylation status of a protein determines its level of activity, especially in signal transduction. When blotting for a post-translational modification, there should always be a control showing the *total* amount of the protein of interest as well. For instance, pT202 ERK1 signal might decrease with a drug treatment. To determine whether this change reflects an alteration in the *proportion* of ERK1 that is phosphorylated (and therefore the proportion of active ERK1), one must be able to determine if the amount of *total* ERK1 present has also changed.

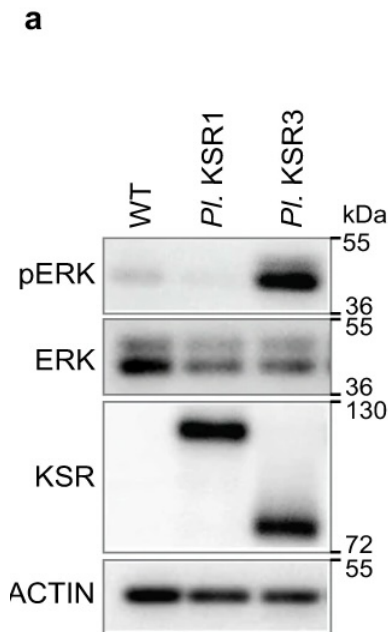


Figure 1. Western blot of HEK293T lysate. Relevant section of caption for published figure reads: “Overexpression of sea urchin KSR3 (Pl. KSR3) but not of KSR1 (Pl. KSR1) triggers ERK signalling in Human HEK293T cells.” “Figure 6” by Chessel et al. ^[1] [\[Image description\]](#)

The authors of this publication knew that in sea urchin development, activation of the ERK pathway is important, but also occurs through a mechanism that is different from the few ways scientists normally see ERK activated. Through an RNA-seq screen, they identified the gene *ksr3* as a candidate for the ERK activator. One experiment they performed to verify this result is shown. They overexpressed sea urchin KSR3 or the related protein KSR1 in human epithelial cells in culture, then made cell lysates for use in the western blot. The researchers wanted to measure ERK phosphorylation, since phosphorylated ERK is the active form. The labels on the top show which sample was run in each lane, whereas the samples on the side show which antibody was used in that piece of the blot. Note that the pERK and ERK antibodies recognize the closely related and similarly sized proteins ERK1 and ERK2, which is why those blots have two closely spaced bands. The top blot shows that there is far more phospho-ERK, i.e. active ERK, in the KSR3-containing sample than the control or the KSR1 sample. The second blot shows that there is actually a decrease in total ERK when KSR1 and 3 are present compared to the control. The third blot uses an antibody that recognizes both KSR1 and KSR3, which are different sizes. This blot just shows that the overexpression was successful. Lastly, actin is a loading control, showing that approximately the same amount of lysate was loaded in each lane. Since there is far more pERK and less ERK in the KSR3 sample compared to the other samples, KSR3 is responsible for activating ERK.

Image Descriptions

Figure 1 image description:

A western blot.

Results of western blot

	WT	<i>Pl. KSR1</i>	<i>Pl. KSR3</i>
pERK	Almost no signal	Almost no signal	Strong signal
ERK	Strong signal, 2 bands	~50% of wt signal, 2 bands	~50% of wt signal, 2 bands
KSR	No signal	Signal further up membrane (larger protein size), similar intensity to KSR3	Signal further down membrane (smaller protein size), similar intensity to KSR1
Actin	Approximately equal signal across all lanes		

↩

Thumbnails

"Rice Genetics III_p789a" ↗ by IRRI Photos is licensed under [CC BY-NC-SA 2.0](#) ↗.

Description: Western blot.

Author

Katherine Mattaini, Tufts University

-
1. Chessel, A., N. De Crozé, M. D. Molina, L. Taberner, P. Dru, L. Martin, and T. Lepage. 2023. RAS-independent ERK activation by constitutively active KSR3 in non-chordate metazoa. *Nature Communications* 14:3970. ↩
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