

Molekulare Pflanzenphysiologie

SS 2014

Script for the lab course
(AG Hoecker)
Interactions between plants
and the environment

part 1

Protokollführung

Verfassen Sie 1 Protokoll pro Gruppe

1. **Einleitung:** Eine kurze Erläuterung der Fragestellung/Versuchsthematik
2. **Methodik** bzw. Messprinzip sollten kurz und knapp erwähnt werden.
3. Der **Ergebnisteil** darf nicht nur Abbildungen mit Ergebnissen enthalten, sondern benötigt einen Fließtext, in dem auf die Abbildungen/Tabellen hingewiesen wird. z.B.: „Abb. 1B zeigt, dass phyB-Mutanten schwächer auf Rc reagierten als wildtypische Keimlinge.“ ODER: „phyB-Mutanten reagierten schwächer auf Rc als wildtypische Keimlinge (Abb. 1B)“.

Wiederholen Sie im Fließtext keine Messdaten, sondern zeigen Sie Trends auf.

Schreiben Sie Ihre Ergebnisse immer in der VERGANGENHEITSFORM (IMPERFEKT)!!!

Schließlich sind das Ihre Beobachtungen und deshalb noch lange keine Tatsachen.

Abbildungen/Tabellen:

- Tabellen und Abbildungen werden separat durchnummeriert. Tabellen erhalten eine **ÜBER**schrift, die Legende zur Abbildung befindet sich **UNTER** der Abbildung.
- Die Legende jeder Abb./Tab. beginnt mit einem Titel, der das Thema der Abb./Tab. beinhaltet (z.B. Hypokotyllängen von wildtypischen und mutanten Keimlingen als Maß für die Lichtantwort). Erst dann folgt die eigentliche Legende.
- Eine Abbildung/Tabelle muss aus sich selbst heraus verständlich sein, d.h. die Legende muss sämtliche Informationen enthalten, die zum Verständnis der Abb./Tab. notwendig sind (z.B. bei einem Gelfoto: was wurde in welcher Spur aufgetragen? Welche Primer wurden verwendet? Wieviel RNA wurde aufgetragen?).
- Besteht eine Abbildung aus mehreren Teilen, werden die Teile mit Buchstaben versehen: A, B, C...

4. Interpretieren Sie Ihre Ergebnisse in der **Diskussion**. Eine reine Fehlerdiskussion reicht nicht. Ordnen Sie Ihre Daten im wissenschaftlichen Kontext ein. Verwenden Sie dazu Informationen aus der Vorlesung, aus Lehrbüchern (Quelle zitieren!) und Publikationen (Quelle zitieren). Bitte auch folgendes beachten, was die Korrektur erleichtert:

5. Bitte keine überflüssigen Wiederholungen der einzelnen Schritte der Versuchsprotokolle, die schon im Skript niedergelegt sind! Es sollten -generell- nur die Zusätze und Abweichungen vom Skript bzw. nicht im Skript niedergelegte Methoden und Arbeitsschritte protokolliert werden.

6. Die Sachverhalte sollten möglichst kurz und präzise dargestellt werden. Auch ein sauber bzw. leserlich von Hand geschriebenes Protokoll wird akzeptiert.

Introduction

One area of current research in molecular biology is the elucidation of signalling pathways. In most cases, model organisms such as yeast, *Drosophila*, mouse and *Arabidopsis* are utilised for this research. The classic signal transduction pathway consists of a receptor, components for signal transduction and transcription factors, which alter gene expression as a response to the signal. The light signal transduction cascade will pose as an example for you to get an insight into the methods and proceedings for the analysis of a signalling pathway.

Green plants harvest energy from sunlight through photosynthesis. Optimised use of sunlight is a crucial criterion, which will determine the chances of survival and reproduction for a plant in competition with its environmental surroundings. Since plants cannot change their location, they have - over the course of evolution - developed methods to adapt their growth and development to their current light conditions in their specific location. This will include, for instance, light-induced germination (as found in some small-seeded species), deetiolation of the seedling in the light (see Fig. 2) and the shade-avoidance reaction (increased length-growth of plants in the shade). Furthermore, light regulates the induction of flower formation through the photoperiod (duration of daytime), and thus the adaptation of plant development to the changing seasons.

To perceive ambient light conditions, plants have a variety of photoreceptors, which react to light of different wave lengths. Among these photoreceptors, the best characterised system is the red/far red-absorbing chromoprotein phytochrome. Charles Darwin already realised that red light triggers a reaction in plants. Later it was shown that this reaction could be reversed by subsequent irradiation with far red light. It became clear that this red/far red antagonism is based on the fact that phytochrome exists in two conformations, which can be reversibly changed from one to the other (Fig. 1): a red-absorbing conformation (P_r , r = red), which is physiologically inactive and is only found under dark conditions, and a far red-absorbing active conformation (P_{fr} , fr = far red). By irradiating with red light, P_r is transformed into P_{fr} ; by irradiating with far red light, P_{fr} is transformed into P_r .

The relative quantities of red and far red in the irradiating light determine the equilibrium between P_r and P_{fr} , and thus the extent of the photomorphogenic response in the plant. Sunlight contains high quantities of red light in comparison to far red light, and therefore acts as red light. On the other hand, light that has been filtered by a canopy of leaves contains high quantities of far-red light, and thus reduces the photomorphogenic response. Hence the phytochrome photoreceptor system allows the plant to perceive other plant populations in their immediate neighbourhood and therefore enables them to adapt their growth and development to this competitive situation.

For the past two decades, molecular exploration of phytochrome function has been of great significance. Active phytochromes initiate a signalling cascade, which leads to an altered expression of a great number of genes. Results from analysing this transduction pathway have enabled scientists to construct a model system (Rockwell et al., 2008).

In the very well explored plant *Arabidopsis thaliana*, five different phytochromes have been identified (A to E, coded by the genes *PhyA* to *PhyE*), of which phytochrome A and phytochrome B exert the most important physiological functions in the plant. The mechanism of signal transfer from phytochrome to the transduction pathway has not been explicitly identified. One interesting fact, however, is that phytochrome has kinase activity, which leads researchers to believe that a phosphorylation reaction might play a part in this signalling pathway.

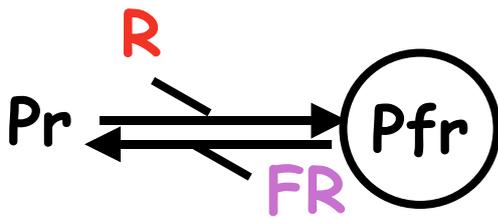


Fig. 1: Phytochrome exists in two conformations, P_r and P_{fr} , which can be reversibly converted from one into the other by red light (R, 660 nm) and far-red light (FR, 730 nm). The P_{fr} conformation is the physiologically active conformation.

References:

Rockwell NC, Su YS, Lagarias JC. (2006) Phytochrome structure and signaling mechanisms. *Annu Rev Plant Biol.* 2006;57:837-58.

A. The genetic approach: Isolation of mutants with a change in light response

A breakthrough in the understanding of phytochrome signal transduction has been accomplished over the last years by isolating mutants with defects in light signal transduction. Seedlings with an altered seedling phenotype were used, since this phenotype may be screened easily and efficiently (see Fig. 2). In principle, two classes of mutants are recognized:

Mutants with a reduced light response. In these mutants, a gene for one of the photoreceptors (e.g. a phytochrome) or for an important positively acting intermediate in light processing (e.g. HY5) is mutated and thus inactive.

Mutants with a constitutive light response. In the dark, these mutants look as if they had been exposed to light. In these mutants, one of the negative regulators for light signal transduction is missing (e.g. COP1), which under normal circumstances is active in the dark and thus prevents occurrence of the "light phenotype" in the dark. If the regulator is mutated, the "light phenotype" will also occur in the dark.

darkness:

- long hypocotyl
- apical hook
- closed cotyledons
- no chlorophyll



light:

- short hypocotyl
- no apical hook
- open, green cotyledons

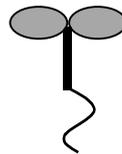


Fig. 2: Phenotype of Arabidopsis seedlings grown in the light or in the dark

Based on characterization of mutants, it was possible to generate a model for light signal transduction (see Fig. 3). The COP1 protein is a central repressor of light signaling and prevents light signaling in darkness. It functions as an E3 ubiquitin ligase which ubiquitinates positive regulators of light signaling, mostly transcription factors, and thereby marks them for degradation in the 26S proteasome. COP1 is primarily active in dark-grown seedlings and thus prevents that these transcription factors activate light-regulated genes also in darkness. In the light, active photoreceptors are thought to inactivate COP1 so that the transcription factors can accumulate.

With the goal to identify new components in the phytochrome signal transduction pathway, new mutations (*spa1*) were isolated, which alter the processing of those light signals perceived by phytochrome (Hoecker et al., 1998). Phenotypic characterization of *spa1* mutants showed that the wild type *SPA1* gene product probably acts as a negative regulator in phytochrome signal transduction.

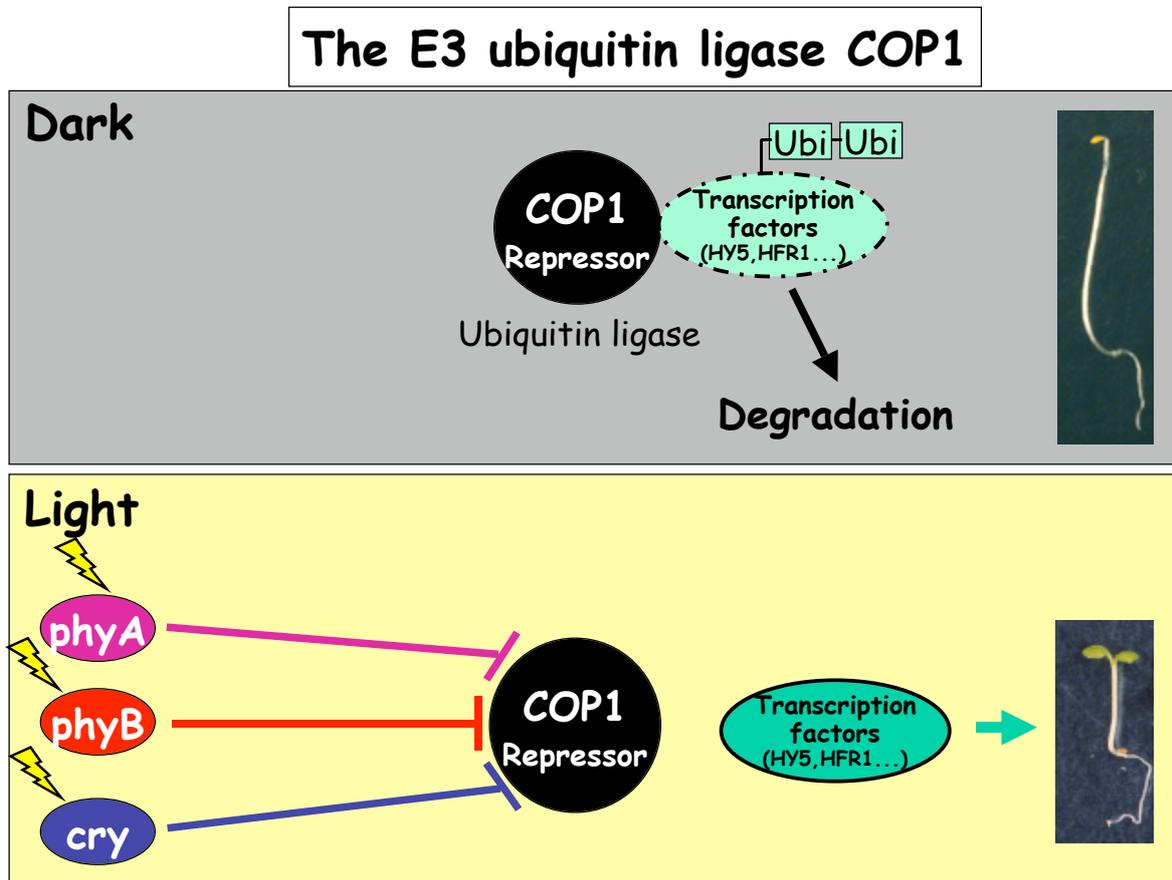


Fig. 3: Model on light signal transduction (explanations see text)

The *SPA1* gene was cloned by positional cloning (Hoecker et al., 1999). It encodes a 115 kD protein which contains different motifs (Fig. 4). In the C-terminal domain, *SPA1* contains two domains - WD-repeats and coiled-coil motifs -, which possibly play a role in inducing interaction with other proteins. Additionally, the *SPA1* sequence shows weak similarity to the kinase motif in serine/threonine kinases and contains two putative nuclear localisation sequences (NLS). Gene expression experiments showed that light strongly increases the amount of *SPA1* transcript.

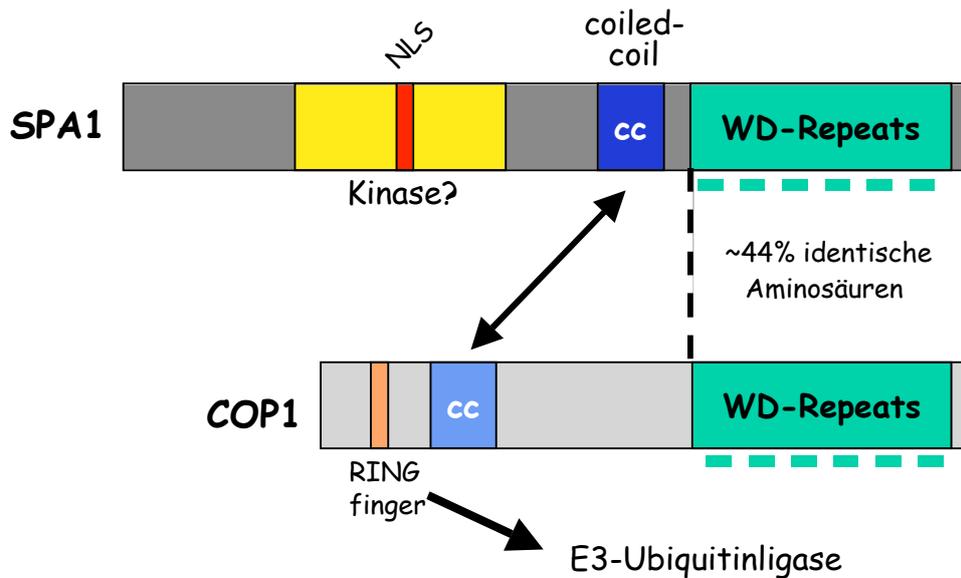


Fig. 4: Structure of the SPA1 protein and its sequence similarity with the repressor COP1

It is notable that the WD-repeat domain of SPA1 shows very high sequence similarity with that of COP1 (Fig. 4). This sequence similarity is limited to the WD-repeat domain since the N-termini of the two proteins are fully distinct. We further found that SPA1 can interact with COP1 via their respective coiled-coil domains. We therefore proposed the model that COP1 acts in concert with SPA1 to ubiquitinate interacting transcription factors. After sequencing of the whole Arabidopsis genome was completed in 2001, we found that *SPA1* is not a single copy gene but that there are three *SPA1*-like genes in Arabidopsis and we named those *SPA2*, *SPA3* and *SPA4*. We subsequently isolated mutants in all of the *SPA* genes. A characterization of these mutants revealed that all four *SPA* genes encode repressors of light signaling. How they act is subject of this lab course.

References:

- Laubinger, S., Fittinghoff, K. and Hoecker, U. (2004). The SPA-quartet: a family of WD-repeat proteins with a central role in suppression of photomorphogenesis in Arabidopsis. *Plant Cell* 16: 2293-2306.
- Laubinger, S., Marchal, V., Gentilhomme, J., Wenkel, S., Adrian, J., Jang, S., Kulajta, C., Braun, H., Coupland, G., and Hoecker, U. (2006). Arabidopsis SPA proteins regulate photoperiodic flowering and interact with the floral inducer CONSTANS to regulate its stability. *Development* 133: 3213-3222.
- Hoecker, U. (2005). Regulated proteolysis in light signaling. *Curr. Opin. Plant Biol.* 8: 469-476.

Tasks:

- Determine the seedling phenotype of different mutants with altered light response. Draw conclusions as to the possible function of the mutated gene in normal light signal transduction.

group	Genotypes	darkness	red light		far-red light		blue light 5 $\mu\text{mol m}^{-2} \text{s}^{-1}$
			30 $\mu\text{mol m}^{-2} \text{s}^{-1}$	1 $\mu\text{mol m}^{-2} \text{s}^{-1}$	5 $\mu\text{mol m}^{-2} \text{s}^{-1}$	0.4 $\mu\text{mol m}^{-2} \text{s}^{-1}$	
1,	WT, <i>phyA</i> , <i>phyB</i>	X	X		X		X
2	WT, <i>cry1 cry2</i>	X	X		X		X
3	WT, <i>cop1-4</i>	X		X		X	X
4	WT, <i>spa1</i> , <i>spa1 spa2 spa3</i>	X		X		X	X
5	WT, <i>spa2 spa3 spa4</i> , <i>spa1 spa3 spa4</i> , <i>spa1 spa2 spa4</i>	X		X		X	X

Solutions and media:

1. Triton X-100

(5 ml for the whole course)

3% (v/v) Triton X-100

Mix Triton X-100 with water and then briefly heat in the microwave to completely dissolve the Triton.

2. Solutions for seed sterilisation

(prepare immediately before use, approx. 1 ml/genotype)

20% (v/v) Klorix (attention: bleaching agent!)
0.03 % Triton X-100 (from stock solution)

3. sterile distilled water

(5x 100 ml, autoclave)

4. MS medium

(2 L for the whole course, autoclave)

4.62 g/l MS salts
Adjust pH to 5.8
Add 10 g/l agar-agar.

Let medium cool after autoclaving and pour into large Petri dishes (15 cm diameter).

Instructions:

1. Aliquot approx. 200 seeds for each genotype into a labelled 1.5 ml Eppendorf tube.
2. Under the tissue culture hood: Add 1 ml of sterilisation solution and invert the Eppendorf tube several times. Incubate the seeds for 10 minutes in sterilisation solution while inverting the tubes occasionally.
3. Wash the seeds 5x with sterile water.
4. Plate the seeds on MS medium. Best to use a 1 ml pipette man.
5. Store the petri dishes at 4 °C in the dark (wrap petri dishes in aluminum foil) for 2 - 4 days. Irradiate the Petri dishes at room temperature with white light for 1 hour. This treatment will break seed dormancy and is necessary for even germination of the seeds. After white light treatment, store the petri dishes at room temperature in the dark for 24 hours, then submit them to red light (660 nm), far red light (730 nm) or darkness (control) for 3 days.
6. Photograph the seedlings with a CCD camera and determine the hypocotyl length with the NIH Image software. Analyse the data (mean and standard error; are differences between the means statistically significant?).

Analysis:

- Compare the phenotypes of the different mutants. Which conclusions can you draw as to the functions of the genes?

B. Generating DNA constructs using Gateway technology

Every molecular biologist needs to generate many recombinant plasmids in order to advance his/her scientific project. These plasmids are for example needed to

- express recombinant protein in E. coli
- generate transgenic plants misexpressing your protein of interest
- conduct a yeast two-hybrid screen
- many, many more ...

The development of the Gateway technology by the company Invitrogen about 8 years ago has greatly facilitated molecular cloning. It has since become a routine method in most molecular biology labs. Other companies have developed similar recombination-based cloning systems. Also non-proprietary cloning systems are available. However, all these systems are used much more rarely when compared to the Gateway system.

What is Gateway?

Here is an excerpt from the company Invitrogen available on the website:

<http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Cloning/Gateway-Cloning/Gateway-Technology.html>

The typical cloning workflow involves many steps, particularly, traditional restriction enzyme cloning. This traditional method limits your cloning success. For example, certain restriction enzymes cannot be used because they might cut within your gene of interest, truncating the insert and making the gene useless for downstream expression.

In contrast, Gateway® recombination cloning technology circumvents these cloning limitations. Gateway is a universal cloning technology based on the site-specific recombination properties of bacteriophage lambda. Gateway® recombination cloning uses a one hour, 99%-efficient, reversible recombination reaction, without using restriction enzymes, ligase, subcloning steps, or screening of countless colonies, thereby saving you time, money, and effort.

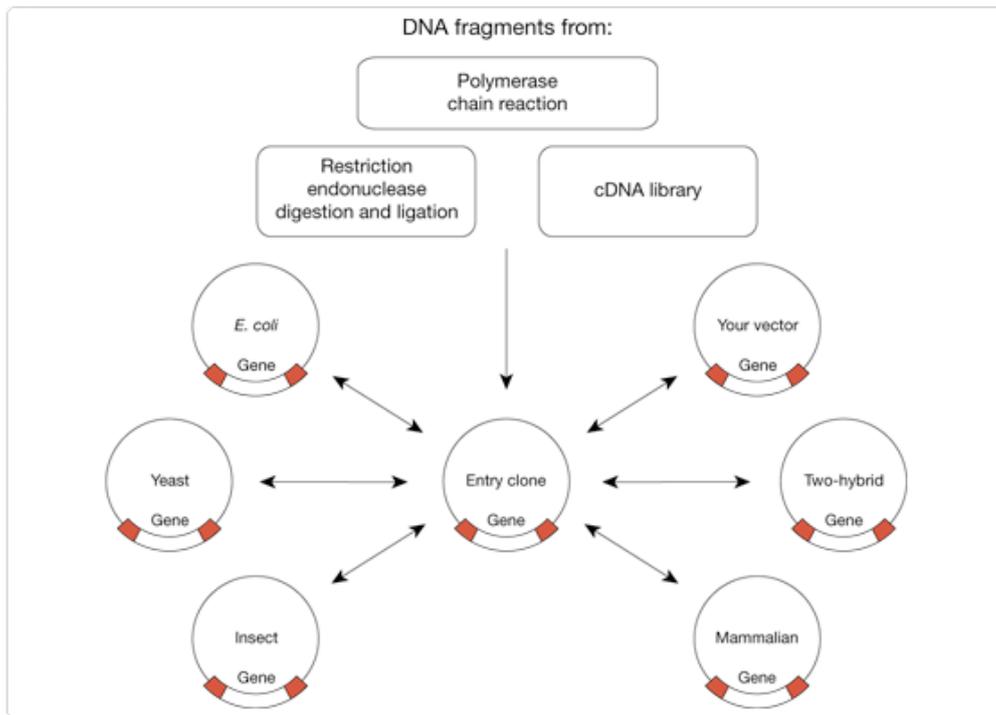


Figure 8. Gateway® technology facilitates cloning of genes, into and back out of, multiple vectors via site-specific recombination. Once a gene is cloned into an Entry clone you can then move the DNA fragment into one or more destination vectors simultaneously.

The essential two parts of Gateway® cloning

Part 1: Constructing an Entry clone.

There are various methods to enter the Gateway® platform. Most widely used is a so-called BP recombination reaction between a pDONR™ vector and an *attB* PCR product containing your favorite gene flanked by the necessary recombination sites (Figures 9, 10). For more information see:

http://tools.invitrogen.com/content/sfs/manuals/gateway_pdonr_vectors.pdf

Part 2: Constructing the expression clone(s) (= Destination vector).

Once the Entry clone is ready, the gene of interest is easily shuttled to a secondary plasmid, the Destination vector of choice. This reaction is mediated by an enzyme mixture called LR Clonase®, which contains the necessary protein activity to excise the gene of interest from the Entry clone and integrate it into the Destination vector, which then becomes your expression clone (Figure 11). Site-specific recombination occurs between *att* sites in the Entry clone and the Destination vector. For more information see:

<http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Cloning/Gateway-Cloning/Clonase-Enzyme.html>

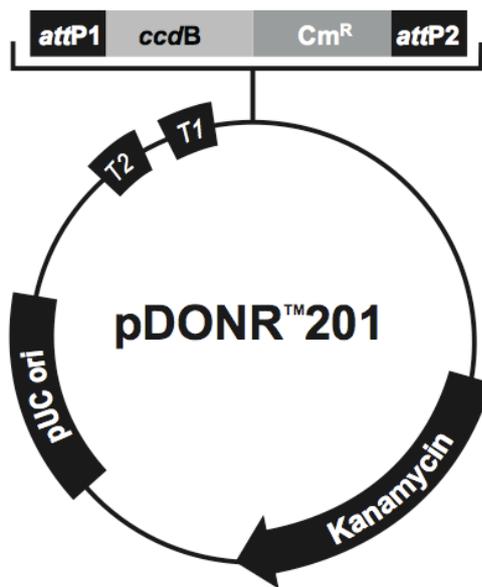


Figure 9: A commercially available pDONR vector.

CCDB gene: suicide gene that allows negative selection against this non-recombined plasmid
 attP1 and attP2: recombination sites

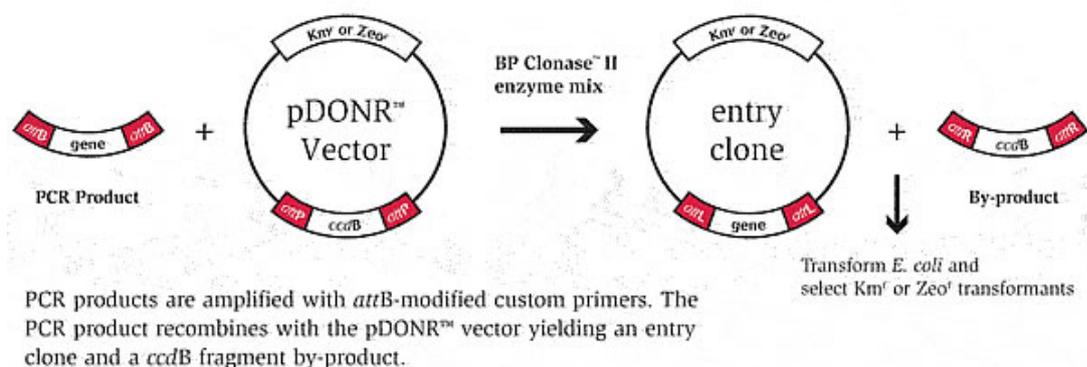


Figure 10: Directional cloning of PCR products into a pDONR vector

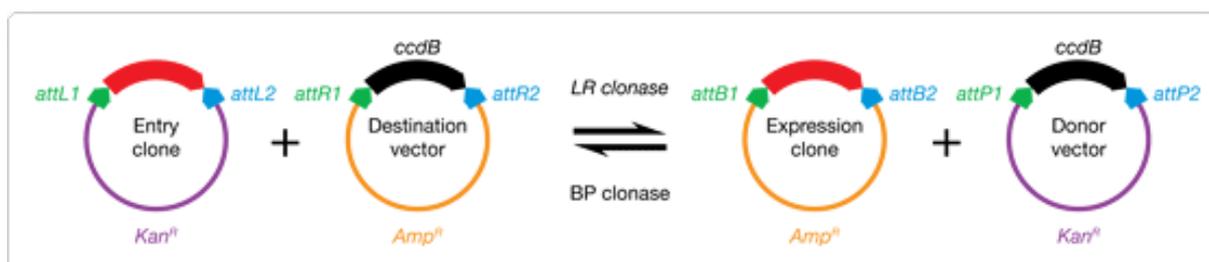


Figure 11: The LR reaction to clone your gene of interest from an Entry clone into the desired Destination clone (= expression vector).

Tasks:

- to perform an LR reaction to generate a Destination vector from a provided Entry/pDONR clone.
- to transform LR reaction into E. coli
- to make plasmid DNA from obtained colonies (Mini prep)
- digest plasmid DNA with appropriate restriction enzymes
- resolve digestion products on agarose gel

Group 1, 2:

Group 3, 4:

Group 5:

Solutions:

1. "Solution 1" (Resuspension solution)

50 mM	glucose
25 mM	Tris (pH 8.0)
10 mM	EDTA (pH 8.0)
add RNase stock solution to a final concentration of 10 $\mu\text{g}/\text{ml}$	

Divalent cations (Mg^{2+} , Ca^{2+}) are essential for DNase activity and the integrity of the bacterial cell wall. EDTA chelates divalent cations in the solution preventing DNases from damaging the plasmid and also helps by destabilizing the cell wall. Glucose maintains the osmotic pressure so the cells don't burst and RNase A is included to degrade cellular RNA when the cells are lysed.
(excerpt from <http://bitesizebio.com/2007/11/07/the-basics-how-alkaline-lysis-works/>.)

2. "Solution 2" (Lysis/Denaturing solution)

0.2 N	NaOH
1%	SDS

The lysis buffer (aka solution 2) contains sodium hydroxide (NaOH) and the detergent Sodium Dodecyl (lauryl) Sulfate (SDS). SDS is there to solubilize the cell membrane. NaOH helps to break down the cell wall, but more importantly it disrupts the hydrogen bonding between the DNA bases, converting the double-stranded DNA (dsDNA) in the cell, including the genomic DNA (gDNA) and your plasmid, to single stranded DNA (ssDNA). This process is called denaturation and is central part of the procedure, which is why it's called alkaline lysis. SDS also denatures most of the proteins in the cells, which helps with the separation of the proteins from the plasmid later in the process.

3. "Solution 3" (Renaturing solution, 3M potassium, 5M acetic acid)

5 M potassium acetate	60	ml
glacial acetic acid	11.5	ml
H ₂ O	28.5	ml

Addition of potassium acetate returns the pH to neutral. Under these conditions the hydrogen bonding between the bases of the single stranded DNA can be re-established, so the ssDNA can re-nature to dsDNA. While it is easy for the small circular plasmid DNA to re-nature it is impossible to properly anneal those huge stretches of E. coli genomic DNA. This is why it's important to be gentle during the lysis step because vigorous mixing or vortexing will shear the gDNA producing shorter stretches that *can* re-anneal and contaminate your plasmid

prep.

While the double-stranded plasmid can dissolve easily in solution, the single stranded genomic DNA, the SDS and the denatured cellular proteins stick together through hydrophobic interactions to form a white precipitate. The precipitate can easily be separated from the plasmid DNA solution by centrifugation.

4. RNAase (10 mg/ml) stock solution

Dissolve 100 mg RNAase A (pancreatic RNAase) in 10 ml 10mM Tris-HCl 15 mM NaCl. Heat to 100°C (in a beaker of boiling water) for 15 min to permanently denature DNases. Cool slowly to room temperature (this allows renaturing of RNase A). Dispense into aliquots and store at -20 °C.

5. 70 % ethanol

6. TE buffer

10	mM	Tris (pH 7.5)
1	mM	EDTA

7. 1 x Elektrophoresis buffer (TBE) (2 L for the whole course)

10,8	g/l	Tris
5,5	g/l	boric acid
0,93	g/l	EDTA

pH should be 8,2 - 8,4 (do not adjust!)

8. Agarose

9. Sample buffer (loading dye) (0,5 ml for each group)

30	%(v/v)	Glycerol
0,25	%(w/v)	Brome phenol blue

do not autoclave, use distilled water

10. Ethidium bromide (will be provided)

5	mg/ml
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final concentration in gel: 0,5 µg/ml

Attention: Ethidium bromide is a powerful mutagen! Wear gloves!!!

Instructions for Gateway LR reactions:

1. Set up the LR reaction and incubate at room temperature (25°C) for at least 1 hr:

Entry clone	ca. 150	ng
Destination vector	ca. 150	ng
5x Clonase reaction buffer	2	µl
LR clonase	2	µl
water or TE buffer	x	µl

C. Generating DNA constructs by traditional cloning

Traditional cloning was always used before the Gateway technology became available around the year 2000. Also today this method is used whenever cloning by Gateway technology is not possible. This is for example the case when specific deletions/additions in a cDNA need to be made. Hence, traditional cloning is still an indispensable technique.

Traditional cloning involves insertion of a piece of DNA into a vector using restriction digestion followed by DNA ligation.

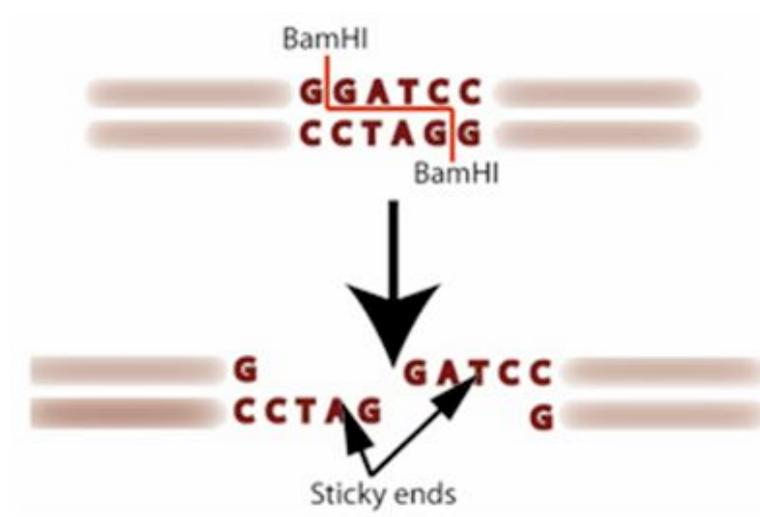


Figure 12: Restriction digestion of DNA with the restriction enzyme BamHI (recognition sequence GGATCC) generates sticky ends.



Figure 13: A restriction fragment (pink) isolated from vector 1 (left) can be ligated into the same restriction sites of vector 2 (right).

(http://academic.wsc.edu/mathsci/christensen_d/bigscam2.html)