TECHNIQUES IN GENETIC ENGINEERING

Işıl KURNAZ

CHAPTER 6

Protein-Protein Interactions

“The ideal engineer is a composite. He is not a scientist, he is not a mathematician, he is not a sociologist or a writer; but he may use the knowledge and techniques of any or all of these disciplines in solving engineering problems.”

N.W.Doherty, 1955

One important feature associated with the function of a protein is its binding partners under different conditions.

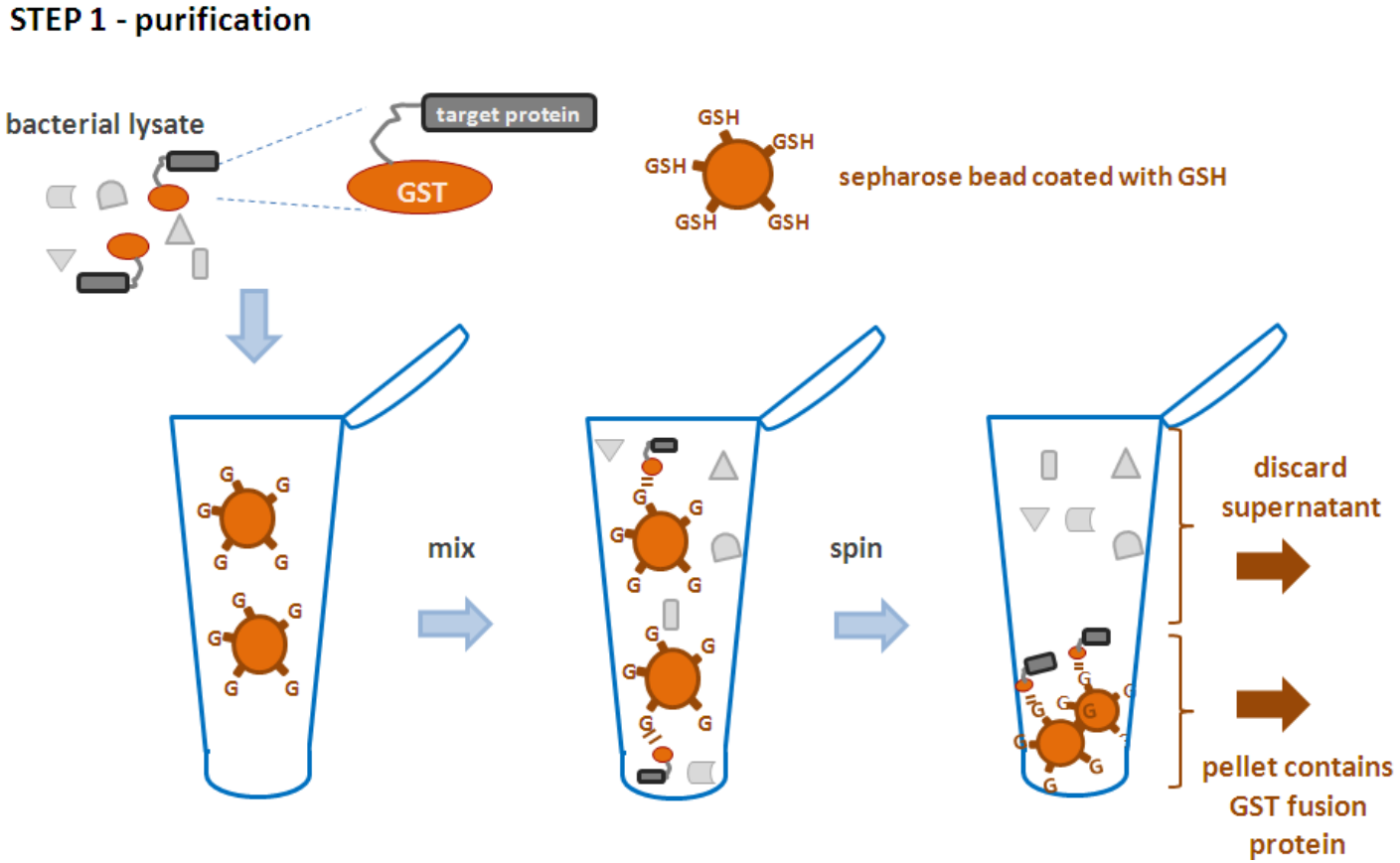
Some proteins have “obligatory” binding partners, without which they cannot function. Some other proteins make “transient” interactions depending on the situation.

No matter what the context, these interactions are fundamentally important for the proper functioning of the cell.

There are many different methods to study these interactions, be it *in vitro* or *in vivo*.

GST pulldown assay

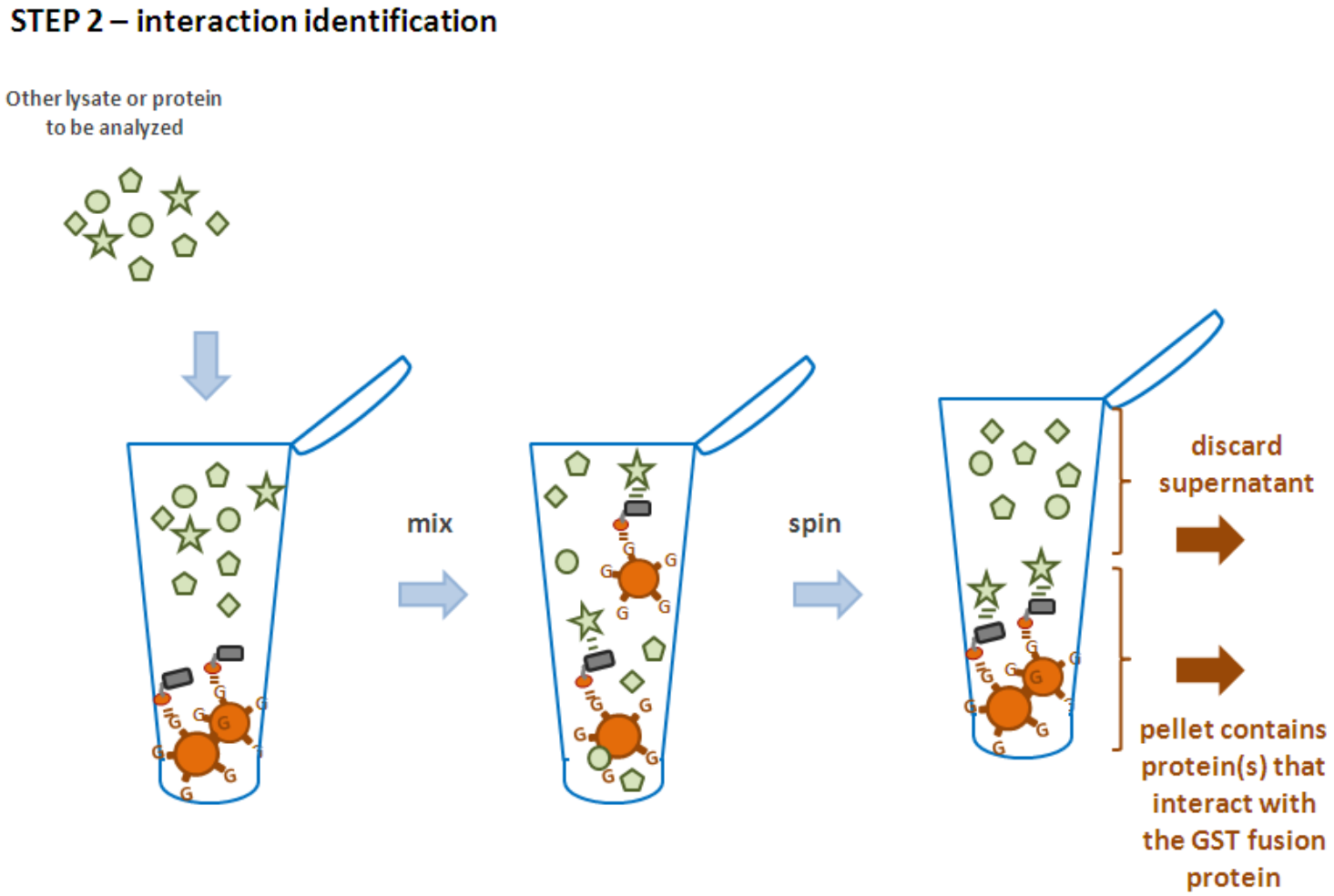
Affinity between glutathione S-transferase enzyme and its substrate, glutathione (GSH), can be used to either semi-purify any protein X that is fused to GST by collecting it on glutathione-beads. In this section, we will not only elute the protein from these beads; but instead, we will use the proteins trapped on the beads to "fish out" any interaction partners, and analyze the presence or absence of the protein we suspect to interact with it.



Care must be taken when cloning GST fusion proteins; START and STOP codons in the coding sequence as well as reading frame must be checked, and expression of the fusion protein confirmed.

Once the presence of the fusion protein in the lysates is confirmed, this lysate is then incubated with glutathione (GSH-) beads for some period, followed by pull-down (hence, the name). The GST-fusion protein will be precipitated with the beads due to enzyme-substrate affinity, along with any other protein that may interact with the GST-fusion protein .

GST pulldown assay



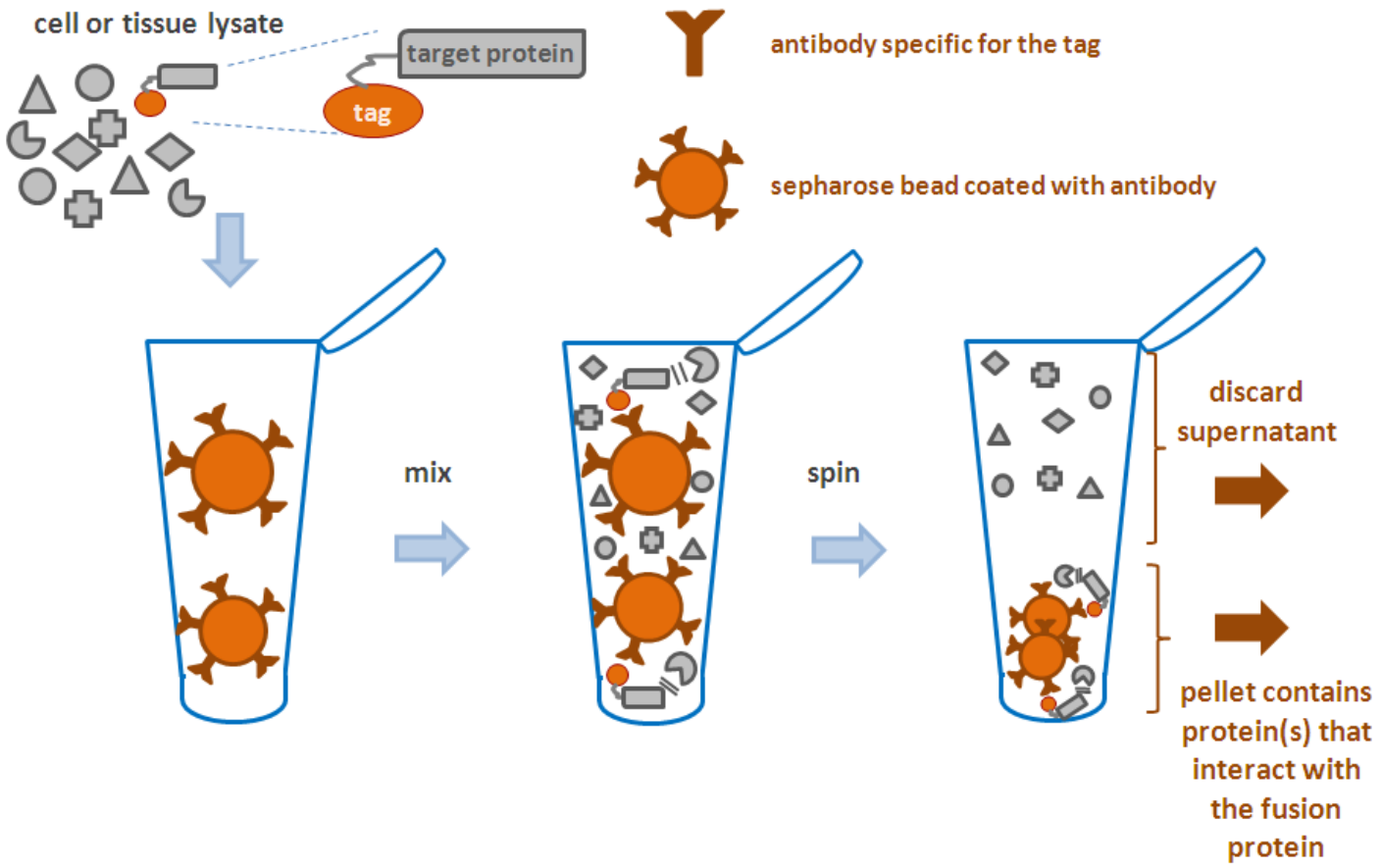
Sometimes a eukaryotic protein may be expressed in bacteria as a GST-fusion for purification purposes. In that case, its interaction partner would be in the eukaryotic cell of origin.

Therefore, in a second step, the pulled-down beads that contain GST-fusion proteins will be incubated with the cell lysate or other purified protein for interaction analysis.

The pellets from the first step will therefore be mixed with this lysate (for example mammalian cell lysate, or mitochondrial homogenate etc), and pulled down a second time.

The second pellet will contain, in addition to the GST-fusion protein than binds to GSH on the beads, any interaction partner of the fusion protein.

Co-Immunoprecipitation (co-IP)



This assay is similar in principle to the GST pulldown assay described before, with the difference that instead of fusion to an enzyme, GST, either the gene of interest is cloned into a tagged-vector (such as pCMV-HA described mostly in this book) and precipitated using antibody-coated beads (ie, immunoprecipitation).

The antigen-antibody affinity will be exploited for precipitating the protein of interest, along with any possible interaction partners (hence, co-immunoprecipitation).

Yeast two-hybrid (Y2H) assay

Yeast two-hybrid system, as well as the mammalian two-hybrid that follows the same principle, relies on the fact that transcription factors are modular, containing a DNA binding domain and an Activation Domain (at minimum) that are functionally separable units; the standard example that is in use in many Y2H vectors is the GAL4 protein in yeast and its cognate *gal4* binding site on DNA.

This assay can either be used to analyze the interaction between two known proteins (let's call them X and Y), or to identify novel interaction partners of a protein X from a cDNA library. The interaction is identified through transcription of a reporter gene, most commonly *lacZ*, and subsequent color reaction using substrate X-gal.

The proteins tested for interaction do not have to be transcription factors or even nuclear proteins at all, any intracellular soluble protein can be tested for interaction.

However the system is not suitable for analysis of membrane protein interactions (a different adaptation of the system is used for that purpose).

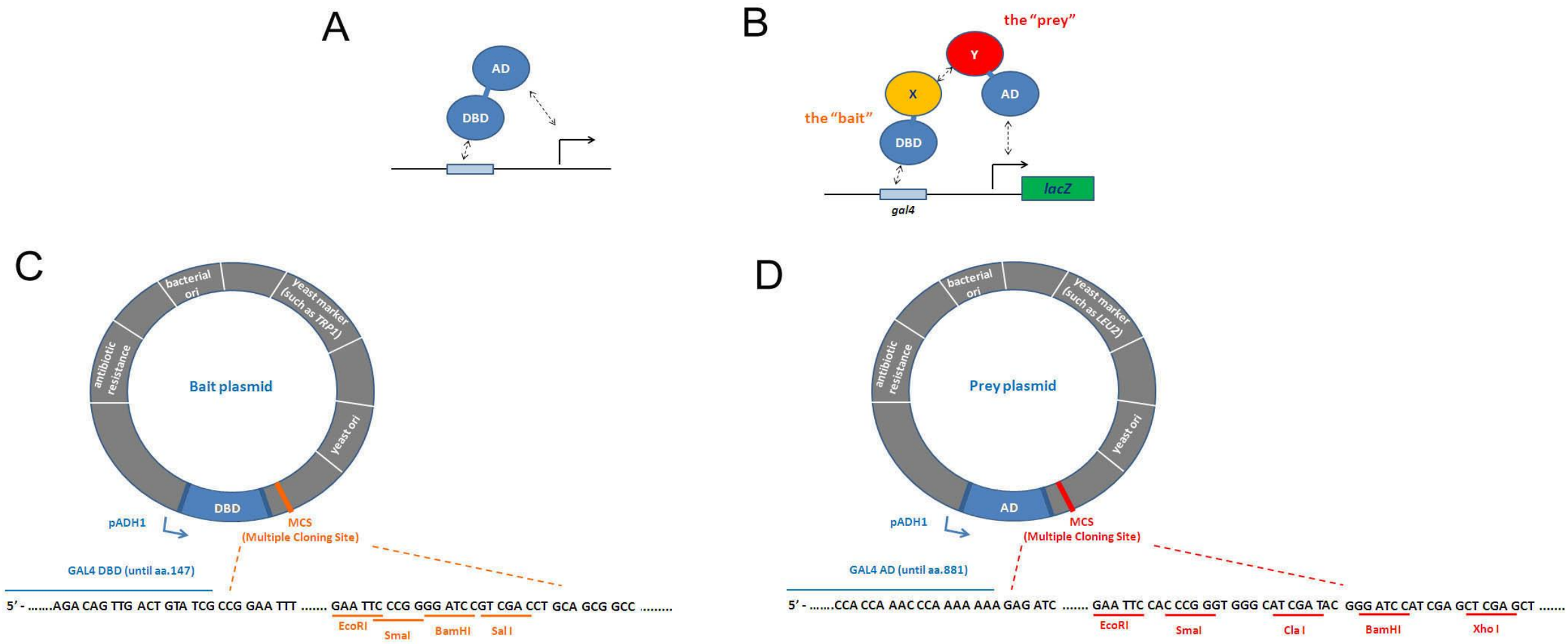


Figure 6.2. A schematic diagram showing the basic principle behind yeast-two-hybrid (Y2H) system. **(a)** the modular structure of transcription factors, with a DNA binding domain (DBD), and activation domain (AD), and a cognate recognition motif on DNA (shown with a box); **(b)** the principle behind the Y2H system, where a protein X is fused to a DBD (typically that of GAL4), and a protein Y is fused to an AD (typically of GAL4), and the interaction between proteins X and Y is monitored through expression of a reporter gene, such as *lacZ*; **(c)** the vector map for a typical bait vector; **(d)** the vector map for a typical prey vector.

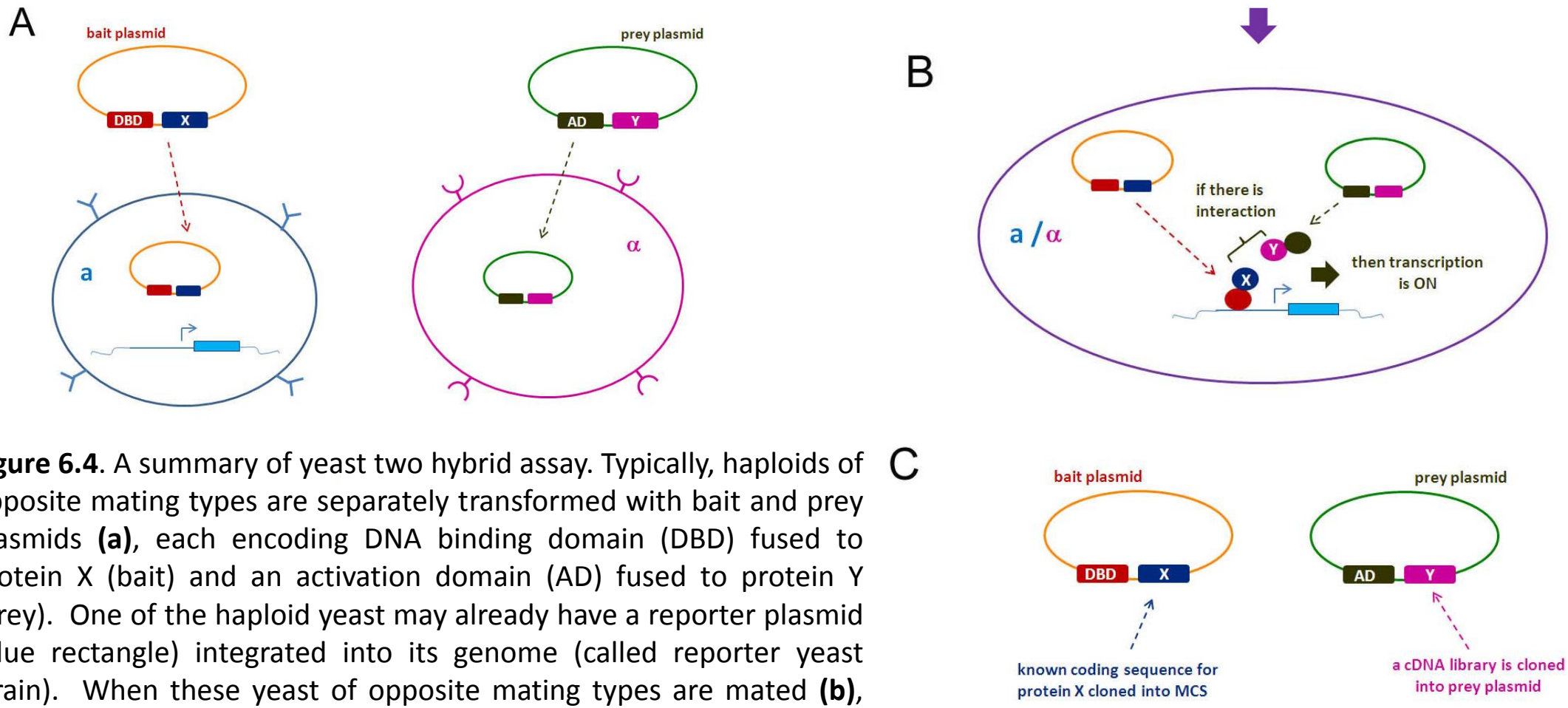


Figure 6.4. A summary of yeast two hybrid assay. Typically, haploids of opposite mating types are separately transformed with bait and prey plasmids **(a)**, each encoding DNA binding domain (DBD) fused to protein X (bait) and an activation domain (AD) fused to protein Y (prey). One of the haploid yeast may already have a reporter plasmid (blue rectangle) integrated into its genome (called reporter yeast strain). When these yeast of opposite mating types are mated **(b)**, the resulting diploid will contain all three components of the yeast two hybrid: bait plasmid, prey plasmid and the reporter gene. If proteins X and Y interact, this interaction will bring the activation domain in close proximity of the promoter element driving reporter gene expression, and transcription will be switched on. **(c)** A cDNA library can be cloned into prey plasmid to carry out an interaction screening for all potential interaction partners of protein X.

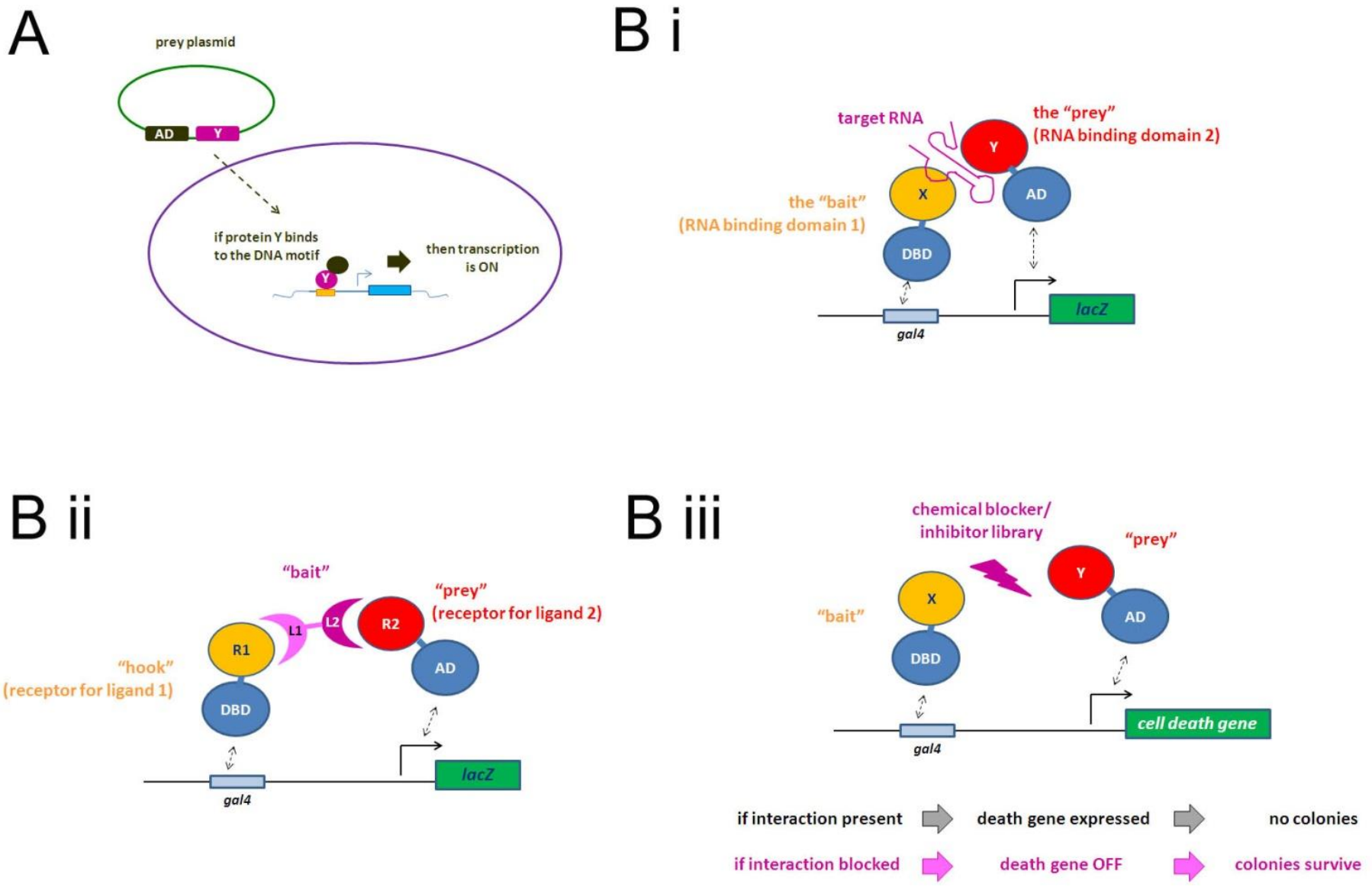


Figure 6.6. Yeast one-hybrid **(a)** and three-hybrid **(b)** systems.
(a) In Y1H, the prey library is screened for any positive interaction with the target DNA binding motif.

(b) Yeast three hybrid can be used for a number of different reasons, such as

(i) identification of three-way interactions between an RNA molecule and two RNA-interacting domains,

(ii) analysis of two different receptors (R1, R2) with either the same (not shown) or different (L1, L2) ligands, or

(iii) screening of a chemical library for potential blockers of interaction between two proteins X and Y. For the latter case, high throughput screening is made easier by the use of a cytotoxic gene (cell death gene) as reporter

Fluorescence Resonance Energy Transfer (FRET)

In vitro interaction assays are highly artificial methods for analysis of protein-protein interactions; yeast or mammalian two hybrid system investigates interactions *in vivo*, however the assay relies on end-point analysis and does not give much information about the dynamic nature of the interactions real-time. Therefore, new methods have been developed for such dynamic studies.

One of the commonly used method for such a dynamic study is the Fluorescence Resonance Energy Transfer (FRET). This method relies on the transfer of resonance energy when two fluorescent molecules are close enough to interact (several angstroms). However, one drawback is that both proteins under investigation have to be fused to different fluorescent proteins for such an energy transfer to occur, which may interfere with either the localization or function of the proteins in some cases.

A

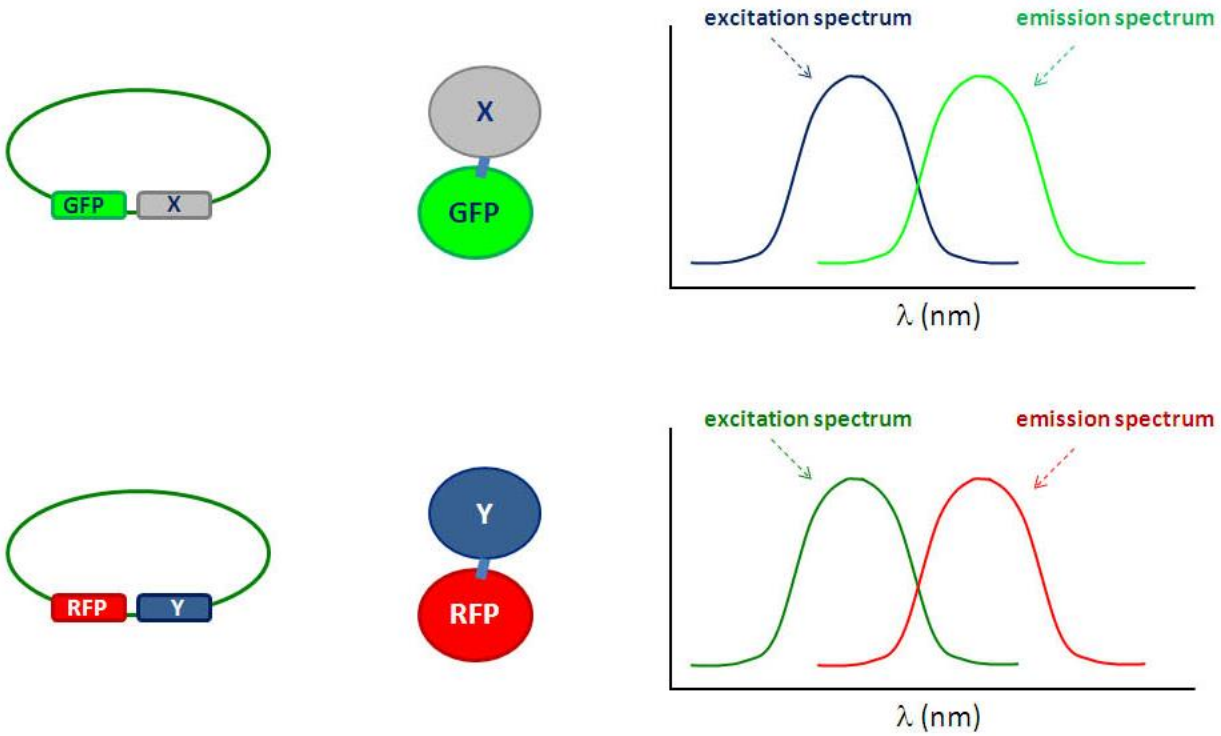


Figure 6.7. An overview of FRET analysis. **(a)** Cloning of both proteins X and Y to be investigated for interaction into appropriate fluorescent expression vectors, pEGFP and pDsRed, respectively (as an example; left panels), the resulting fusion proteins (middle panels), and the respective excitation and emission spectra (right panels)

B

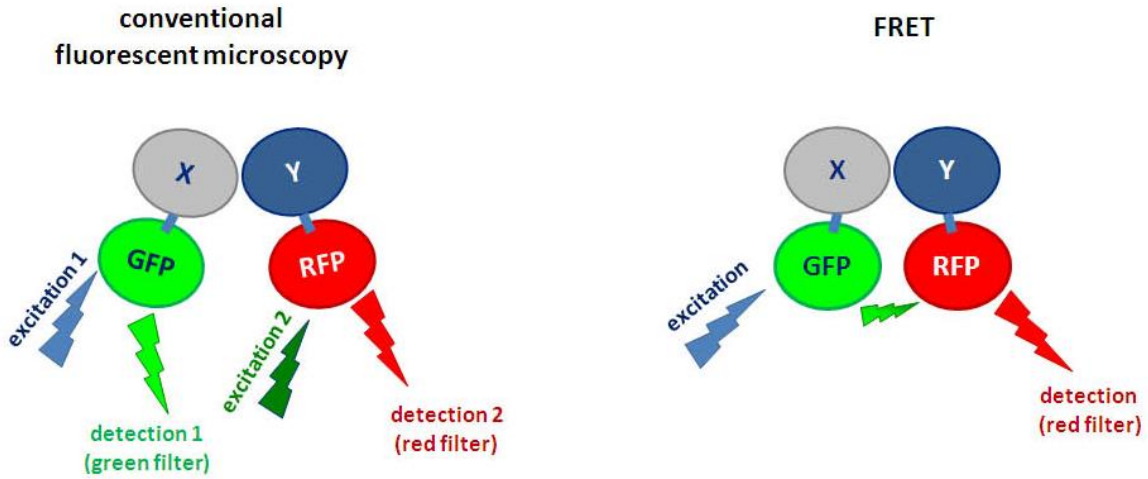


Figure 6.7. An overview of FRET analysis.

(b) comparison of excitation and detection of fluorescence in conventional microscopy (left panel) vs FRET (right panel);

C

Fluorescent Protein	Excitation (nm)	Emission (nm)
mPlum	590	649
mCherry	588	610
DsRed	566	586
mOrange	548	586
EYFP	514	527
EGFP	488	527
mCFP	433	475
T-Sapphire	399	511

(c) a sample table summarizing excitation and emission peaks (filters in fluorescent microscopes) of some common fluorescent proteins