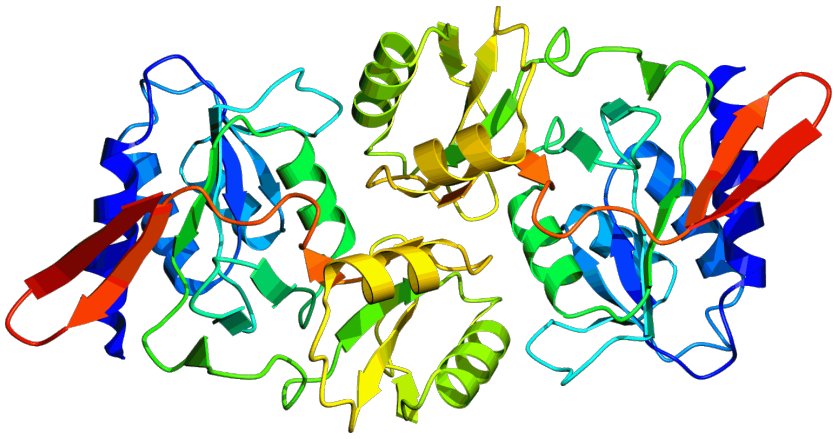
Site-directed Mutagenesis of Ribose 5-Phosphate Isomerase (RpiA)

Lab protocol: Protein Engineering course 2023



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Introduction

The protein studied in your lab work is part of an on-going research project at Uppsala University, and earlier, at SLU. It is likely that no one has ever made the mutations that you will introduce. The scientists involved will use the information you gather in their research, so we are all very excited to see the outcome! This comes with a great deal of responsibility. Since your results will be integrated into real research, you have to be very careful, and be sure you record (take notes on) everything you do. Furthermore, we will ask you to present your work to the rest of the class before and after the wet lab (in English).

The lab work is advanced, and you will be using methods and equipment that are used on a daily basis in modern research laboratories. You will use structural and other information about ribose-5-phosphate isomerase (RpiA) to choose which amino acids you want to mutate; furthermore, you will be asked to predict the outcome, *i.e.* the effects on the enzyme's activity and/or other properties. Then you will design the oligonucleotides necessary to introduce the mutations. The next step is to actually introduce the mutation/s into the gene (*rpiA*) encoding RpiA by *site-directed mutagenesis*. For this you will need to do *PCR*, followed by *transformation* and *plasmid preparation*. Furthermore, you have to verify *protein expression* by running *SDS-PAGE*. The enzyme will then be purified by *metal-chelating chromatography*, desalted by *gel filtration chromatography* and *concentrated*. Finally you will measure the enzyme's activity by using *an assay based on spectrophotometry…*and all this in just two and a half weeks! Since we are short of time, the lab days will often be long and you should take this in account when you plan the rest of your life (outside the lab).

Risks and protection

Chemicals:
· Ampicillin (Amp). Antibiotic. Wear suitable protective clothing.

· Dimethyl sulfoxide (DMSO). In case of contact with eyes, rinse immediately with plenty of water. Wear suitable protective clothing.

· Hydrochloric acid 1M (HCl). Causes severe burns. In case of contact with eyes, rinse immediately with plenty of water, contact the hospital and have someone take you there. Wear suitable protective clothing, gloves and eye/face protection.

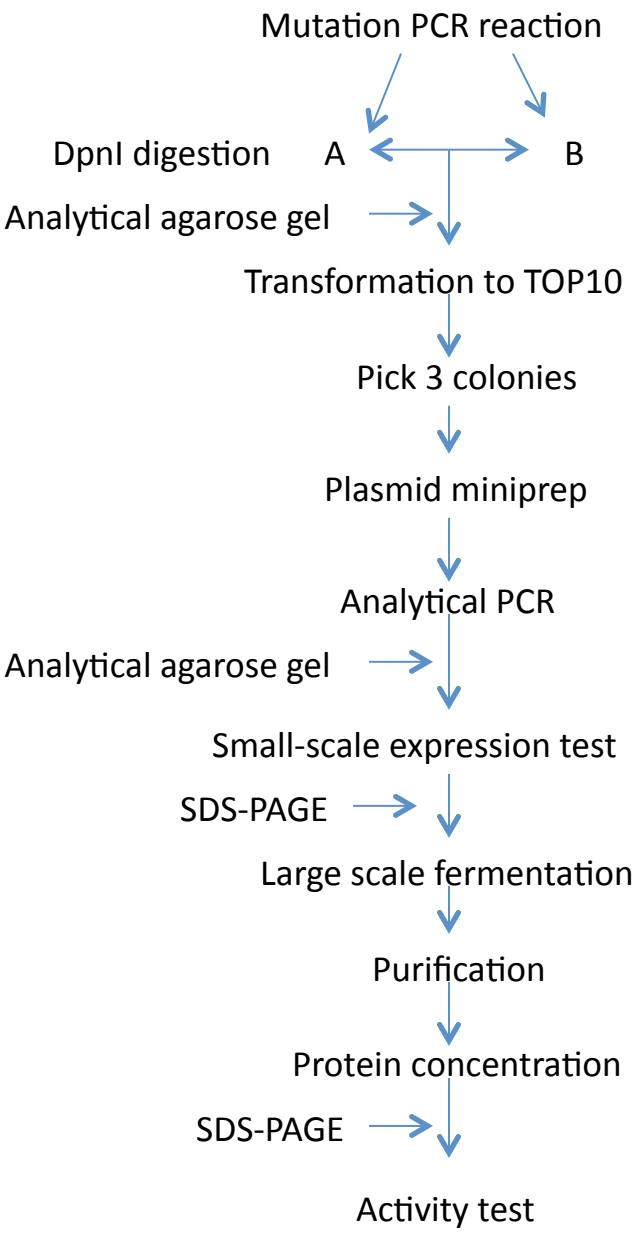
· Imidazole. Corrosive. In case of contact with eyes, rinse immediately with plenty of water. Wear suitable protective clothing, gloves and eye protection.

· Sodium dodecyl sulfate (SDS). Denatures proteins. In case of contact with the eyes or skin wash immediately with water.

Organisms:
· Bacteria. *Escherichia coli* (TOP10).

Other:
· Polyacrylamide gel. In case of contact with eyes, rinse immediately with plenty of water. Wear suitable protective clothing.

Overview


Mutation PCR reaction
DpnI digestion
A
B
Analytical agarose gel
Transformation to TOP10
Pick 3 colonies
Plasmid miniprep
Analytical PCR
Analytical agarose gel
Small-scale expression test
SDS-PAGE
Large scale fermentation
Purification
Protein concentration
SDS-PAGE
Activity test

Instructions on how to prepare lab presentations (and report)

All students will give two presentations concerning the lab.

The first will outline the mutations to be made (complete with a description of the structural setting of each), and the expected effects on the protein's properties (activity, stability, etc.).

The second will remind the audience of the goals with the mutations, and describe the results obtained, and their interpretation. For this purpose, it is essential that all items are shown and clearly identified, all gel lanes are labelled, molecular weight markers are included, etc., etc., just like in real life! Details of how these presentations will be graded are found on the course web pages.

We will also ask you to hand in your own lab notebook for grading. Make sure you start using your notebook, starting with the day mutations are chosen, and keep it up to date during the rest of the lab-related events. Instructions on how that will be graded can be found on the course web site. "Useful" definitely ranks higher in our thoughts than "beautiful", but "tidy" can go a long way toward that "useful" goal.

L1: Design of mutants, primers and ordering of primers

To design oligonucleotides for your PCR, you need the DNA and protein sequences. You also need a codon usage table, so that you can select a codon frequently used by *Escherichia coli*; this will help you to get good expression. This information is given below.

To introduce the mutation with the PCR method, you will need two oligos that, a) contain the mutation and b) are complementary to each other. A third shorter oligo will be used in a PCR hybridization analysis.

The following example shows how you can approach the oligo design problem. Suppose we want to mutate of H79 to N. We can choose to do this through a single base change, i.e. CAT to AAT. Other possibilities should also be considered. One should preferably introduce mutations that create high frequency codons.

Wild type

```
71 I  P  L  S  V  L  D  D  H  P  R  I  D  L  A  I  D  G  A  D
  ATTCCGCTCTCCGTTCTCGATGATCATCCTCGAATTGACCTCGCCATTGATGGCGCCGAT
```

mutant

```
71 I  P  L  S  V  L  D  D  N  P  R  I  D  L  A  I  D  G  A  D
  ATTCCGCTCTCCGTTCTCGATGATAATCCTCGAATTGACCTCGCCATTGATGGCGCCGAT
```

1. We need two complementary oligonucleotides with the mutation in the center. The oligos should include about 18 nucleotides on each side of the mutation (max. length of oligo = 46 nt) and the GC content about 40-60% (optimally 50%). If possible, the first and last nucleotide of the oligos should be G or C. Our first mutation oligo, G1.H79N1 (where G1 in this pretend case stands for Group 1), thus has the following sequence:

5’ CTCTCCGTTCTCGATGATAATCCTCGAATTGACCTCG 3’

2. The second oligo is an exact complement to G1.H79N1. To write the complementary oligo in the 5' to 3' direction, it is convenient to make use of the link http://www.basic.northwestern.edu/biotools/oligocalc.html; the oligo you want is generated when you click on “Calculate”.

5’ CGAGGTCAATTCGAGGATTATCATCGAGAACGGAGAG 3’

3. The melting temperature (T_m) of your mutation oligo can be calculated using the following formula:

$T_m = 81.5 + 0.41(\%GC) - 675/N - \%mismatch$ (T_m should be ~78°C)

where N is the length (in nucleotides) of your oligo.

Calculate the T_m for your mutagenesis oligos using the formula above.

4. Finally, our analytical hybridization oligo (in this case G1.H79N3) is:

5’ CTCTCCGTTCTCGATGATA 3’

This oligo will be used in an analytical PCR together with another oligo (either RpiA forward or RpiA reverse, which are complementary to the 5' or 3’ end of the *rpiA* gene, respectively; more

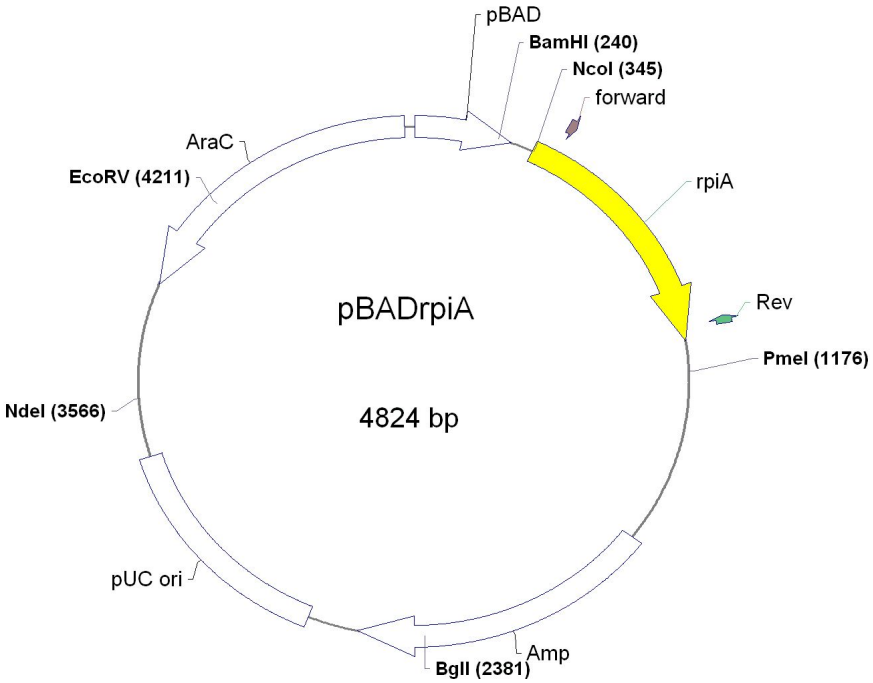
on this below). With the help of these two oligos, we can amplify the DNA, but only if the mutation was successful.

5. Calculate the melting temperature (T_m) of your analytical primer using the Primer design software available at http://www.basic.northwestern.edu/biotools/oligocalc.html. Type in your oligo sequence in the "Enter Oligonucleotide Sequence" box, click on "Calculate", and view the results below. The melting temperature calculations give you three different values. We are going to use the Tm values marked as "(Salt Adjusted)". The "help" section of the page explains the assumptions used for the calculations; please read it. It is recommended that you design your analytical primers to have a Tm ~58°C. You can use the same tool to check the self-complementarity of your primer.

6. Send your oligos by e-mail to Sanjeewani (sanjee.soori@icm.uu.se) with the following information included for each of the three oligos:

Group (*e.g.* G1)
Mutation (*e.g.* D6A)
Oligo id (what you want to call your oligo, e.g. G1.H79N1)
T_m

The map for the plasmid you will use, pBADrpiA, is shown below: The *rpiA* gene, location for primers (rpiA forward and reverse) and selected unique restriction sites (bold) are indicated.



RpiA protein sequence (amino acids of the mature protein are shown in boldface, please use this numbering at all times!).

```
        M   G   S   H   H   H   H   H   H
   CCC ATG GGA TCT CAT CAT CAT CAT CAT CAT

1  G   V   L   T   Q   D   D   L   K   K   L   A   A   E   K
   GGA GTC TTA ACT CAA GAC GAT CTC AAG AAA CTC GCC GCC GAA AAA

16 A   V   D   S   V   K   S   G   M   V   L   G   L   G   T
   GCC GTC GAC TCC GTC AAA TCC GGC ATG GTT CTC GGT CTC GGA ACC
```

```
31  G   S   T   A   A   F   A   V   S   R   I   G   E   L   L
    GGA AGT ACT GCC GCA TTT GCT GTC TCG CGA ATC GGC GAG CTT CTC

46  S   A   G   K   L   T   N   I   V   G   I   P   T   S   K
    TCT GCC GGA AAA CTG ACC AAC ATC GTT GGA ATT CCT ACC TCG AAG

61  R   T   A   E   Q   A   A   S   L   G   I   P   L   S   V
    CGG ACC GCA GAG CAG GCG GCG TCT CTT GGA ATT CCG CTC TCC GTT

76  L   D   D   H   P   R   I   D   L   A   I   D   G   A   D
    CTC GAT GAT CAT CCT CGA ATT GAC CTC GCC ATT GAT GGC GCC GAT

91  E   V   D   P   D   L   N   L   V   K   G   R   G   G   A
    GAG GTT GAT CCT GAT CTT AAT CTG GTT AAG GGG CGC GGT GGG GCG

106 L   L   R   E   K   M   V   E   A   A   S   D   K   F   I
    CTC TTG AGA GAA AAG ATG GTT GAA GCT GCT AGT GAT AAA TTT ATT

121 V   V   V   D   D   T   K   L   V   D   G   L   G   G   S
    GTT GTT GTT GAT GAT ACT AAG CTT GTT GAT GGT TTG GGT GGT AGT

136 R   L   A   M   P   V   E   V   V   Q   F   C   W   K   Y
    CGT CTT GCT ATG CCT GTT GAA GTT GTT CAA TTT TGC TGG AAA TAT

151 N   L   K   R   L   Q   E   I   F   K   E   L   G   C   E
    AAT CTC AAG AGA TTA CAG GAG ATC TTT AAG GAG CTG GGT TGT GAG

166 A   K   L   R   M   E   G   D   S   S   P   Y   V   T   D
    GCA AAA TTG AGA ATG GAA GGG GAT AGC AGT CCT TAT GTG ACT GAC

181 N   S   N   Y   I   V   D   L   Y   F   P   T   S   I   K
    AAC TCG AAT TAC ATC GTG GAT TTA TAC TTC CCG ACC TCG ATT AAG

196 D   A   E   A   A   G   R   E   I   S   A   L   E   G   V
    GAT GCT GAA GCT GCA GGG AGA GAA ATT TCG GCC TTG GAA GGC GTA

211 V   E   H   G   L   F   L   G   M   A   S   E   V   I   I
    GTA GAA CAT GGG TTG TTC TTG GGT ATG GCT AGC GAA GTC ATC ATT

226 A   G   K   T   G   V   S   V   K   T   K   -
    GCT GGG AAA ACT GGA GTT AGT GTG AAA ACC AAG TGA
```

rpiA gene (including His-tag)

```
     CCCATGGGAT CTCATCATCA TCATCATCAT
  1  GGAGTCTTAA CTCAAGACGA TCTCAAGAAA CTCGCCGCCG AAAAAGCCGT
 51  CGACTCCGTC AAATCCGGCA TGGTTCTCGG TCTCGGAACC GGAAGTACTG
101  CCGCATTTGC TGTCTCGCGA ATCGGCGAGC TTCTCTCTGC CGGAAAACTG
151  ACCAACATCG TTGGAATTCC TACCTCGAAG CGGACCGCAG AGCAGGCGGC
201  GTCTCTTGGA ATTCCGCTCT CCGTTCTCGA TGATCATCCT CGAATTGACC
251  TCGCCATTGA TGGCGCCGAT GAGGTTGATC CTGATCTTAA TCTGGTTAAG
301  GGGCGCGGTG GGGCGCTCTT GAGAGAAAAG ATGGTTGAAG CTGCTAGTGA
351  TAAATTTATT GTTGTTGTTG ATGATACTAA GCTTGTTGAT GGTTTGGGTG
401  GTAGTCGTCT TGCTATGCCT GTTGAAGTTG TTCAATTTTG CTGGAAATAT
451  AATCTCAAGA GATTACAGGA GATCTTTAAG GAGCTGGGTT GTGAGGCaAA
501  ATTGAGAATG GAAGGGGATA GCAGTCCTTA TGTGACTGAC AACTCGAATT
551  ACATCGTGGA TTTATACTTC CCGACCTCGA TTAAGGATGC TGAAGCTGCA
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```
601  GGGAGAGAAA TTTCGGCCTT GGAAGGCGTA GTAGAACATG GGTTGTTCTT
651  GGGTATGGCT AGCGAAGTCA TCATTGCTGG GAAAACTGGA GTTAGTGTGA
701  AAACCAAGTG A
```

RpiA forward oligo sequence: 5'-CTCAAGAAACTCGCCGCCGAA-3'

RpiA reverse oligo sequence: 5'-CCAGCAATGATGACTTCGCTA-3'

Table of *Escherichia coli* codon usage

From Hénaut and Danchin: Analysis and Predictions from *Escherichia coli* sequences. *Escherichia coli* and *Salmonella*, Vol. 2, Ch. 114:2047-2066, 1996, Neidhardt FC ed., ASM press, Washington, D.C. Genes are clustered by using factorial correspondence analysis into three classes. Class I contains genes involved in most metabolic processes. Class II genes correspond to genes highly and continuously expressed during exponential growth. Class III genes are implicated in horizontal transfer of DNA. One can see that the distribution of codons in class III genes is more or less even, whereas it is extremely biased in class II genes (in particular, codons terminating in A are selected against).

Amino Acid	Codon	Class				Amino Acid	Codon	Class		
		I	II	III				I	II	III
Phe	ttt	55.09	29.08	67.14		Leu	ctt	9.70	5.56	19.00
	ttc	44.91	70.92	32.86			ctc	10.40	8.03	9.04
Leu	tta	10.99	3.44	20.09			cta	3.09	0.83	6.81
	ttg	13.02	5.47	15.05			ctg	52.79	76.67	29.99
Ser	tct	13.26	32.41	19.63		Pro	cct	13.71	11.23	28.30
	tcc	15.02	26.56	11.34			ccc	11.19	1.63	16.26
	tca	10.83	4.79	22.09			cca	18.63	15.25	31.50
	tcg	16.88	7.39	10.60			ccg	56.47	71.89	23.94
Tyr	tat	54.42	35.23	69.60		His	cat	56.80	29.77	61.69
	tac	45.58	64.77	30.40			cac	43.20	70.23	38.31
Stop	taa					Gln	caa	33.40	18.65	37.06
	tag						cag	66.60	81.35	62.94
Cys	tgt	40.90	38.85	55.71		Arg	cgt	38.99	64.25	26.05
	tgc	59.10	61.15	44.29			cgc	42.23	32.97	21.94

Stop	tga						cga	5.52	1.07	12.80
Trp	tgg	100.00	100.00	100.00			cgg	8.97	0.80	13.62
Ile	att	51.20	33.49	47.57		Val	gtt	23.74	39.77	34.33
	atc	44.37	65.94	26.65			gtc	22.48	13.45	18.95
	ata	4.43	0.57	25.78			gta	14.86	19.97	21.78
Met	atg	100.00	100.00	100.00			gtg	38.92	26.81	24.94
Thr	act	14.85	29.08	26.83		Ala	gct	14.52	27.54	22.86
	acc	46.83	53.60	24.45			gcc	27.62	16.14	23.67
	aca	10.52	4.67	27.93			gca	19.63	24.01	31.27
	acg	27.81	12.65	20.80			gcg	38.23	32.30	22.19
Asn	aat	40.87	17.25	64.06		Asp	gat	62.83	46.05	70.47
	aac	59.13	82.75	35.94			gac	37.17	53.95	29.53
Lys	aaa	75.44	78.55	72.21		Glu	gaa	68.33	75.35	66.25
	aag	24.56	21.45	27.79			gag	31.67	24.65	33.75
Ser	agt	13.96	4.52	18.73		Gly	ggt	32.91	50.84	31.79
	agc	30.04	24.33	17.61			ggc	43.17	42.83	24.51
Arg	aga	1.75	0.62	15.63			gga	9.19	1.97	24.75
	agg	1.54	0.29	9.96			ggg	14.74	4.36	18.95

L2: Start PCR mutation

In this experiment, you will introduce your mutation into the *rpiA* gene with the help of a PCR reaction, according to the QuikChange Site-Directed Mutagenesis Kit manual. As DNA template, you will use the pBAD_RpiA expression vector, and the two oligonucleotides you designed that carry the mutation will be used as primers for the reaction.

Materials:
PCR tubes
PCR machine
Pfu Ultra polymerase (2.5 units/µl)
Pfu reaction buffer (10x)
Oligo no 1 (diluted to 10 pmol/µl)
Oligo no 2 (diluted to 20 pmol/µl)
Plasmid pBAD_RpiA: pBAD plasmid (4083 bp) plus *rpiA* (741 bp) = total size of 4824 bp
dNTP stock solution (10 mM of each nucleotide)

Sterile water
The QuikChange Site-Directed Mutagenesis Kit manual

Procedure:
The PCR program is described below. You will run two experiments for each mutation in parallel: **A** is your main experiment and **C** is a control reaction in which *Pfu* Ultra is not added

PCR program:
95°C 2 min
95°C 45 s
55/60°C 1 min } 18 cycles
68°C 5 min
(1 min/1000 bp)
4°C 99 h

Reaction mixture:
Keep everything on ice, except for the *Pfu* Ultra enzyme, which should be kept in a freezing block. It is a good idea to bring the buffer to room temperature, and mix it well, before adding it (some salts may have precipitated out during freezing, for example). Add the ingredients in the order described below, and mix the reaction components by tapping the tube after addition of the last ingredient.

A	**C**
40 µl H_2O	41 µl H_2O
5 µl buffer (10x)	5 µl buffer
1 µl plasmid (50 ng)	1 µl plasmid
1 µl oligo 1 (10 pmol)	1 µl oligo 1
1 µl oligo 2 (20 pmol)	1 µl oligo 2
1 µl dNTP (10 mM)	1 µl dNTP
1 µl *Pfu* Ultra (2.5 U)	-

L3: Analysis of mutation PCR

In L2, you performed the PCR mutation reaction. In order to verify that the plasmid has actually been amplified (hopefully introducing the mutation), you will do an analysis of the PCR product by agarose gel electrophoresis. Before you do this, you will digest the parent plasmid DNA with *Dpn*I (which only digests methylated DNA, *i.e.* it will digest your template plasmid, but not your PCR product).

Materials:
*Dpn*I restriction enzyme
50x TA / Safe SYBR (10000x in DMSO)
DNA loading dye
Agarose
GeneRuler DNA size marker (500-10 000 bp)

Procedure:
1. Keep the PCR tubes on ice.

2. In experiment **A**, take a sample of 10 µl from tube A and transfer it to a new tube, which you call B. Add 1 µl *Dpn*I to tube A. Incubate at 37 °C for 1 h. Leave tube B on ice during this time.

3. During digestion, prepare the agarose gel (see recipe below). Several groups can share the same gel.

4. Prepare samples from each of the experiments, i.e. A, B and C. Pipette a sample of 8 µl from each tube (save the rest in the freezer) and add 2 µl dye solution. Apply the entire volume on the gel. **Note: Make sure that you have at least 3 µl left in the A tube (no dye added) after you have withdrawn samples to load on the gel** – you will need this for the transformation**.**

5. Apply 10 µl of GeneRuler DNA size marker in one lane of the gel. Run the gel at 50 mA for about 30 min.

6. Store all PCR reactions (including the *Dpn*I treated sample) in the freezer (-20 °C).

7. View the resulting gel under UV: take photos and save with your lab notes, making sure you have **complete and correct labels (this applies to all gels you run)**. Remember that this result, like all those following, will be included in your report. It is a lot easier to do it now, than to remember "what was what" later!

Agarose gel
You will analyze the PCR product on a 0.9 % agarose gel. (For optimal choice of gel concentration in other cases, see "Molecular cloning: a laboratory manual" by Sambrook et al.). Prepare the gel form by closing the ends of the tray with tape, and put the comb in place. Weigh 1.8 g agarose into a glass flask and add 200 ml 1x TA. Boil in the microwave oven. Cool the agarose solution by swirling; be careful not to produce too many bubbles. When the solution has reached about 50 °C (= when it is still hot, but not too hot to hold), add 20 µl Safe SYBR solution (stock solution 10 000 x) to a final concentration of 1x (1 µL SYBR/10 ml agarose solution) and pour it out in the gel form. Let the gel solidify on the bench.

Transformation
You will transform your mutated plasmids, and the wild type pBAD_RpiA vector as a control (positive control for transformation and protein expression, negative control for the analytical PCR), into your competent cells.

Materials:
Luria-Bertani agar medium (LA) plates
Ampicillin (Amp) stock solution (50 mg/ml)
LB medium

1. Use 25 µl of TOP10 competent cells for each transformation. Thaw the frozen cells on ice (takes about 5 min). Add 1 µl of *Dpn*I-treated mutation PCR reaction product (A) or 1 µl of untreated mutation PCR reaction product (B), as appropriate, to each tube. (Some groups will also be asked to transform some cells with the wild type pBAD_RpiA vector using 0.1 µg DNA.)

2. Place the tubes on ice for 5 min.

3. Give the cells a heat shock. This is done by placing the tubes in a 42 °C heating block for 30 s. Put the tubes back on ice for 2 min.

4. Add 100 µl LB to the transformed cells and spread on LA-Amp plates. Be gentle when spreading the cells! (If the transformed plasmid had carried a gene for antibiotic resistance other than ampicillin, we would have had to perform phenotypic expression before spreading on the plates, by incubation at 37 °C for 1 h.)

5. Seal the plates with Parafilm, and place them upside down at 37 °C overnight.

L4: Inoculation of overnight cultures

In this part of the lab, you will inoculate 2 ml LB-Amp cultures with single colonies from the plates then incubate the cultures overnight. The cells will then be harvested, and the plasmids isolated by a mini-prep procedure. (Overnight cultures are frequently used for plasmid preparations, but when, for example, cloning toxic genes, it can be safer not to allow the culture to remain at stationary phase for such a long time.)
An alternative method to organize and pick your colonies after the transformation procedure is to use "patching". This allows colonies obtained in a random disposition on one or several plates to be represented on a single petri plate. This procedure will be shown in the lab.

Materials:
13 ml round bottom tubes or 15 ml Falcon tubes
LB
Ampicillin stock solution (50 mg/ml)
LA-Amp plates

Procedure:
1. Add 2 ml LB + 50 µg/ml ampicillin to each of 7 Falcon tubes.

2. Select 3 single colonies per mutant, preferably with different sizes, from your mutant plates. Pick each colony with a sterile yellow tip or sterile toothpick, touch the surface of an LA-Amp plate, then use to inoculate a 2 ml culture. Inoculate the seventh tube with a colony from the pBAD_RpiA control plate.

3. Place the tubes at an angle in a stand and put in the shaker at 37 °C overnight.

L5: Mini-prep and agarose gel analysis

Plasmid mini-preps can be done in several ways, some of which make use of small columns. In this lab you will use the Qiagen spin columns. The method starts with disruption of the bacterial cells at alkaline pH with SDS in the presence of RNase. SDS, bacterial chromosomal DNA and proteins are precipitated with high salt, and pelleted along with the cell debris. The plasmid remains soluble, and is bound to a resin at high ionic strength, then eluted in 10 mM Tris-HCl pH 8.5. All centrifugations are done at maximum speed in an Eppendorf centrifuge.

Materials:
QIAprep Spin Miniprep Kit
Agarose
50x TA

Safe SYBR (10 000x in DMSO)
DNA loading dye

Procedure:
1. Cast an agarose gel (0.9 %).

2. Transfer the 2 ml cell cultures to 2 ml Eppendorf tubes. Save 50 µl from each clone for the expression experiments (store in the refrigerator, i.e. at 4 °C).

3. Collect cells by centrifugation at top speed for 1 min. Remove supernatant.

4. Resuspend cells in 250 µl Buffer P1 (from the kit) by vortexing or pipetting. (Make sure that RNaseA has been added to the P1 buffer.)

5. Add 250 µl Buffer P2. Mix by inverting 6 times.

6. Add 350 µl Buffer N3. Mix by inverting 6 times. Centrifuge for 10 min.

7. Withdraw 750 µl of the supernatant and apply to the spin column tubes.

8. Centrifuge for 1 min at top speed. Discard the flow-through.

9. Wash the column with 750 µl Buffer PE. Centrifuge for 1 min at top speed. Discard the flow-through, and centrifuge the column again for 1 min.

10. Place the column in a clean 1.5 ml Eppendorf tube. Add 50 µl Buffer EB. Wait 1 min before centrifugation for 1 min at top speed.

11. Perform agarose gel electrophoresis to analyze the plasmid preparation. Take 2 µl from each clone and add 6 µl H_2O and 2 µl dye. As markers on the gel, put 10 µl GeneRuler DNA size marker, as well as samples containing 50 ng and 100 ng of the template pBAD_RpiA (wild type) plasmid from the first PCR. Estimate the concentration of your plasmid preps by comparing the gel bands to those of the template plasmid samples.

12. Determine your DNA concentration by measuring the absorbance at 260 nm. Take 2 µl of the plasmid preparation and load it directly in the nanodrop spectrophotometer. Another alternative is to prepare a dilution 1:25 (2 µl of the plasmid + 48 µl of autoclaved distilled water) and measure the DNA concentration using the microcuvettes of the Biophotometer instrument (1 OD_{260} = 50 µg/mL of dsDNA).

L6: Verification of mutants by PCR

Hybridization is a frequently used method for identification of DNA or RNA. In this experiment you will use a PCR-based hybridization analysis to distinguish between the mutant and wild type clones. The shortest primer that you designed (“N3”) contains the mutation, and will, at an appropriate temperature, only anneal properly to the mutant DNA, and be extended. This oligo

will act as one primer in the PCR. Depending on where in the *rpiA* gene your mutation is located, the second primer in your PCR will be either "RpiA forward" or "RpiA reverse", which bind to the 5' and 3' end of the *rpiA* gene, respectively. It is convenient to choose the primer combination that produces the largest band. *Taq* polymerase is 10 times cheaper than *Pfu* Ultra, and it will therefore be used here, since we do not need the proofreading activity of *Pfu* Ultra in this step.

Materials:
Taq DNA polymerase (1 unit/µl)
Taq reaction buffer (10x)
dNTP (10 mM of each nucleotide)
Primers (diluted to 20 pmol/µl)

PCR reaction:
1. Start by programming the PCR machine!

94°C 2 min
94°C 45 s,
*55°C 45 s. } 20 cycles
72°C 45 s
(45 s for 1 kb, 1 min for 1.5 kb, 2 min for 3 kb; how long is your PCR product? See sequence map.)
4° C 99 h
* The temperature to be used depends on the T_m of the oligo; it is typically 5 °C below the calculated T_m

2. Label five PCR tubes for each mutation. Tubes 1-3 are for your experimental samples, 4 is a negative control, and 5 is a positive control.

3. Place 50-100 ng of your candidate clone DNA (1-3) in the bottom of PCR tubes 1-3. Place 1 µl of pBAD_RpiA (wild type) in each of the control tubes 4 and 5.

4. Prepare a PCR master mix for tubes 1-4:
- 208 µl H_2O
- 5 µl dNTP
- 25 µl buffer (10x)
- 2.5 µl analytical primer
- 2.5 µl RpiA forward **OR** Rpi reverse (**which one should you use?**)

5. To tube 5, add:
41.5 µl water
1 µl dNTP
5 µl buffer (10x)
0.5 µl RpiA forward
0.5 µl RpiA reverse

6. Add 2.5 µl *Taq* polymerase to your master mix (see step **4**) immediately before the PCR reaction is to be started. Mix by pipetting (vortexing will kill the enzyme). Pipette 49 µl of master mix into PCR tubes 1-4.

7. Add 0.5 µl *Taq* polymerase to tube 5, mix gently by tapping.

L7: Verification continued: Analysis of PCR products by agarose gel electrophoresis.

The PCR products from the analytical PCR will now be analyzed by agarose gel electrophoresis. The primers were designed such that they would only be extended if the plasmid is mutated. This is true at the optimal annealing temperature. If you cannot see a clear difference between the wild-type negative control and your candidate clones, you should run the analysis again, and this time try annealing at 60 °C.

Materials:
50x TA
Safe SYBR (10 000x in DMSO)
DNA loading dye/glycerol
Agarose
DNA size marker (100 bp ladder, 100-2000 bp)

First: start cultures for test expression of your protein (L8)!

Agarose gel
Analyse the PCR products on a 1.5 % agarose gel (3 g agarose in 200 ml 1x TA) with 1 µl SYBR/10 ml agarose solution. Apply the samples (8 µl PCR product + 2 µl dye) and a size marker (100 bp ladder). View the results under UV light, take photos and save with your lab notes; make sure you have added complete and correct labels for the various lanes on the gel.

L8: Analysis of clones by small-scale test expression experiments.

Theoretically, all clones should be identical, and express the protein equally efficiently. In practice, this is often not the case. For example, we might have damaged the expression vector in the mutation PCR, or the recombinant cells might have picked up a mutation that makes it harder for them to cope with the plasmid. You will therefore do test expressions of all of the positive clones identified in the analytical PCR (L6 and L7), *i.e.* where your mutagenesis has been successful. You do not have to transform the plasmid to a new cell line for expression - you can just continue with the bacterial culture that you have saved (step 2 of L5). As a positive control in the expression experiment, you will use the wild-type pBAD_RpiA culture (only for selected groups).

Materials:
6 or 7 culture tubes (some groups will run an additional positive control)
LB
Ampicillin (50 mg/ml)
L-Arabinose (200 mg/ml, 100x) **Note that D-arabinose will not work!**

Disposable UV cuvettes

Procedure:
1. Inoculate 3.5 ml LB + ampicillin (50 μg/ml) in 13 ml round bottom tubes with 25 μl of the saved bacteria from each of the six clones. Start the inoculum in the morning. As a positive control for expression, some groups will use pBAD_RpiA (wild type) in tube 7.

2. Just before induction (see step 3), remove 300 μl cells, centrifuge at 11,000 rpm for 1 min, discard supernatant and save the pellet in the freezer (-20 °C). OBS - also take out 100 μl cells and save in refrigerator. These cells will be used to inoculate overnight cultures tomorrow (L9).

3. After lunch, or when the OD_{600} ≈0.7 (use 0.5 ml culture mixed with 0.5 ml LB for OD_{600} measurement, → >2 ml left for expression), induce expression by addition of L-arabinose to a final concentration of 2 mg/ml.

4. Let the cells grow for 2.5 h.

5. After completed expression, measure OD_{600}.

6. Take a sample corresponding to 500 μl of culture at OD_{600} = 1 (e.g. if OD_{600} = 2, then take 250 μl).

7. Centrifuge the sample at 13,000 rpm for 1 min in an Eppendorf centrifuge. Remove supernatant and store the cell pellets in the freezer till the next day (-20 °C).

L9: Analysis of test expression by SDS gel electrophoresis, selection of clone for large-scale expression, and start of inoculum for large-scale expression.

Materials:
5 x SDS sample buffer
1 x SDS sample buffer
SDS gels and running buffer (1 g SDS, 3 g Tris base, 14.4 g glycine)
Low Molecular Weight (LMW) protein marker
Instant Blue stain
LB
Ampicillin (50 mg/ml)

Procedure:
1. Suspend each sample (yesterday's cell pellets) in 50 μl 1x SDS-sample buffer.

2. Heat-denature at 95 °C for 5 min. Vortex vigorously for 1 min to shear cellular DNA (if sample is still slimy after vortexing, vortex longer).

3. Assemble the electrophoresis equipment.

4. Load 10 μl of the samples, plus the size marker, LMW (10 μl, mixed 1:1 with 2x SDS sample buffer), on the gel.

5. Run the electrophoresis at 10 V/cm.

6. Stain the gel with Coomassie and leave it at room temperature for 30 min. Destain (with pads) at room temperature.

7. Take photos of the gel. As always, label everything carefully!

8. Select two clones for each mutation, one that you are going to use for the large-scale experiment and one as a backup in case the first one does not grow for some reason.

9. Inoculate 5 ml LB + ampicillin (50 μg/ml) in a 50 ml Falcon tube with 20 μl of the saved cells from each selected clone. Incubate at 37 °C overnight.

L10: Scaled up fermentation of mutant clones

Materials:
LB (pre-heated)
Ampicillin stock (50 mg/ml)
L-Arabinose stock (200 mg/ml, i.e. 100x stock solution)

Procedure:
1. Inoculate 100 ml LB + ampicillin (50 μg/ml) with 2 ml of the overnight culture (in a 500 ml flask). Incubate at 37 °C on shaker. **Be careful when placing the flasks on and removing them from the shaker table, they can break!**
Use another 2 ml of the overnight culture to prepare plasmid template for sequencing (plasmid prep performed as L5).

2. When OD_{600} has reached about 0.7:
a) Take a sample corresponding to 500 μl at OD_{600}=1 (e.g. if OD_{600} is 0.5, take 1 ml), centrifuge 13,000 rpm 1 min, remove supernatant and store the pellet at -20 °C (-> sample 1).
b) Induce expression with arabinose at a final concentration of 2 mg/ml.

3. Put the flask back on the shaker at 37 °C for 3 h.

4. After completed expression, measure the OD_{600}.

5. Take a sample corresponding to 500 μl at OD_{600} = 1 (e.g. if OD_{600} = 2, then take 250 μl), centrifuge at 13,000 rpm for 1 min, discard the supernatant and store the cell pellets at -20 °C (→ sample 2).

6. Harvest the cells by centrifugation (swing-out rotor, 4000 rpm at 4 °C for 20 min) in 2 x 50 ml Falcon tubes. Discard the supernatant and store the cell pellets at -20 °C.

L11: Isolation and purification of isomerase.

Our isomerase protein is provided with a 6xHis-tag. This usually makes it possible to obtain quite pure protein in one step, by making use of a nitrilotriacetic acid (NTA) column with immobilized Co^{2+} ions. (Nickel columns are also commonly used.) The histidines will form a complex with the Co^{2+} ions. The protein can then be eluted with a buffer containing a high

concentration of imidazole (elution with histidine can also be used, in cases where imidazole is problematic), which will compete with the histidine residues for the binding to the Co^{2+} ions. But first we have to lyse the bacteria, to get the protein out! Here we will do that by addition of lysozyme in combination with vortexing. All purification steps should be carried out on ice, where possible.

Changes in the protein sequence (i.e. mutations) can affect the folding of a protein. In addition, high concentrations of the protein can produce aggregates. The formation of inclusion bodies often results, so you should be on the lookout for this during the expression and purification of your protein. Carefully analyse your gels and expression levels with this in mind!

Materials:
Co-NTA 50% slurry
Lysis buffer: 0.5% Triton X-100, 50 mM Tris-Cl pH 7.5, 300 mM NaCl
Wash buffer: 50 mM Tris-Cl pH 7.5 and 300 mM NaCl
Elution buffer: 50 mM Tris-Cl pH 7.5, 300 mM NaCl, 150 mM imidazole
Columns
Lysozyme/DNase/RNase solution

Procedure:
1. Resuspend the cell pellet in 10 ml lysis buffer/Falcon tube (20 ml in total). You will have one tube for each mutant (or wild type).
2. Add 2 ml of a solution containing lysozyme (50 mg/ml), DNase (0.05 mg/ml), and RNase (0.1 mg/ml) and incubate at 37 °C for 15 min.
3. Vortex lysate for 20 sec.
4. Centrifuge at 4000 rpm for 30 min.
5. Transfer the supernatant to a new 50 ml tube. Save a 40 µl sample of the supernatant at -20 °C for SDS gel analysis (→ sample 3).
6. Save the pellet at -20 °C. (Some of your protein might be in this fraction, in the form of inclusion bodies.)
7. Add 1 ml of 50% Co-NTA slurry to the supernatant and mix gently on a tilting table at 4 °C for 30 min.
8. Load the lysate-Co-NTA mixture into a column. Let the column material sediment before unscrewing the cap (you will have to load it twice).
9. Save a 40 µl sample of the flow-through at -20 °C for SDS gel analysis (→ sample 4). Also save the rest of the flow-through at -20 °C. Your protein might be in this fraction for some reason!
10. Wash the resin with 2 x 4 ml wash buffer. Collect wash fraction and save it at -20 °C.
11. Elute the protein with 2 ml elution buffer. Collect the eluate and save 40 µl at -20 °C for SDS gel analysis (→ sample 5).

L11: Continued: Desalting and concentrating your protein.

When measuring the activity of the isomerase, we need to get rid of additives (*e.g.* imidazole) that can interfere with the kinetic measurements. This is accomplished by running your protein sample through a desalting column (which works on the principle of gel filtration).

Next, the protein needs to be concentrated to reach a suitable working concentration for the activity measurements. This is accomplished by spinning the desalted protein solution through a

membrane with an appropriate pore size that will let the solution go through but not the protein.

Desalting

Materials:
PD-10 desalting columns (Sephadex G-25 Medium)
Sample buffer: 50 mM Tris pH 7.5

Procedure:
1. Remove top cap of the desalting column and pour off excess liquid.

2. Remove bottom cap.

3. Equilibrate the gel bed with 25 ml sample buffer.

4. After running sample buffer through the column, add your sample (2.5 ml) to the column. (Note – if sample volume is less than 2.5 ml, add sample buffer to your sample until a total volume of 2.5 ml is achieved.) Discard the flow-through after filtration.

5. When the sample has run into the column, elute your protein with 3.5 ml sample buffer. Save 40 µl of the desalted protein solution at -20 °C for SDS gel analysis (-> sample 6).

6. Measure the protein concentration of your sample (see L12). If the protein concentration of your sample is higher than 5 mg/mL, skip steps 1-3 of the concentration procedure (see below).

Concentration

Materials:
Vivaspin concentrator (cut-off 10 kDa)

Procedure:
1. Transfer your protein sample to the concentrator. Make sure that the screw closure is fully seated.

2. Insert the assembled concentrator into the centrifuge (if fixed angle rotors are used, turn the concentrator so that the printed window faces upwards/outwards).

3. Centrifuge at 3000 g (4000 rpm in "Ci" centrifuge with swinging bucket) or at 7500 g (fixed angle).

4. Once the desired volume is achieved (corresponding to 5-10 mg/ml, if possible), remove assembly and recover sample from the bottom of the concentration chamber with a pipette. **This is your (hopefully) pure enzyme that you will save at 4 °C. Do not freeze it!** Remove 10 µl of the concentrate for SDS gel analysis (-> sample 7).

L12: Analysis of purification by SDS-PAGE and determination of yield

We have already used the method of SDS-PAGE (SDS polyacrylamide gel electrophoresis) for analysis of the expression of RpiA. We will now use this method for analysis of the purification by the metal chelating chromatography, desalting and concentration. You have saved aliquots from the cell pellets before and after induction, from the supernatant after cell lysis (you should run the pellet, too, if inclusion bodies are suspected, from the flow-through and eluate from the Co^{2+} column, from the desalted protein solution, and from the concentrated protein (samples 1-7).

Materials:
SDS 5x sample buffer
SDS 1x sample buffer
SDS-gels and running buffer (1g SDS, 3g Tris base, 14.4 g glycine)
LMW protein marker
Coomassie Brilliant Blue (or AquaStain) stain and destain solutions

Procedure:
1. Add 50 µl 1x SDS sample buffer to the pellets (sample 1 and 2). Add 10 µl 5x SDS sample buffer to the 40 µl samples (samples 3 to 6). Add 2.2 µl 5x SDS sample buffer to the 10 µl sample (sample 7).
2. Heat-denature at 95 °C for 5 min.
3. Assemble the electrophoresis equipment.
4. Load 10 µl of the samples (sample 1-7), plus the size marker (LMW, 10 µl), on the gel.
5. Run the electrophoresis at 100 V (10 V/cm).
6. Add Coomassie to the gel, then leave it at room temperature for 30 min. Destain at room temp. Take photos of the gel, and label as usual!

<u>Determination of yield and protein concentration</u>

Calculate the protein concentration by measuring the absorbance at 280 nm (turn on the UV light in advance) and using the Lambert-Beer law: $A_{280} = \varepsilon \times l \times c$
where ε is the **extinction coefficient of the protein** (in this case 11 460 $M^{-1}cm^{-1}$, the estimate one gets from ProtParam assuming all cysteines are reduced), l is the cuvette length in cm, and c is the concentration of protein in the cuvette (molar). For the Nanodrop machine, a correction is automatically made to give the value appropriate for a 1 cm path length.

L13: Assay for ribose-5-phosphate isomerase (RpiA)

Reaction and physical basis of the assay
The reaction catalysed by RpiA is

ribose 5-phosphate $\rightleftharpoons$ ribulose 5-phosphate

The fact that ribulose 5-phosphate absorbs UV light at 290 nm, and ribose 5-phosphate doesn't, means that one can measure the reaction in either direction very simply, by following the increase or decrease of absorbance at 290 nm. Here, we'll use ribose 5-phosphate as substrate,

measuring the increase in absorbance at 290 nm. (We will use quartz cuvettes or special plastic ones for this, as glass or most plastic ones would absorb the UV light.)

Common mistakes can be avoided!
Remember to:

- Dilute your protein in buffer. Diluting it with water could kill it.
- Keep your protein on ice, and inspect it during the day to see any aggregation is visible. If you suspect anything is wrong, make a fresh diluted protein sample.
- Mix well using Parafilm to cover the cuvette. Make sure there are no air bubbles, scratches or fingerprints on the surface of the cuvette, but be as fast as you can.
- Be careful that no dust gets into the cuvette, e.g. from the Parafilm.
- When you have a very low substrate concentration, you will need to be very fast mixing and starting the measurement, since the equilibrium will be reached very quickly.
- Keep track of how much protein and substrate you have, so you don´t run out. There might be a little more substrate (if you are lucky), but the protein is all you have, and it is important to get points below your K_m, as well as near V_{max}.
- Think about what you expect from your mutant(s) when planning the experiment. Should Km or kcat be influenced, how could that influence the way you do your measurements? But also look at your own results; don´t be blinded by what you expect.
- Read the manual first, if you have questions, then ask the teachers. That will save you time in the end!

Materials:
General stock solutions
1 M Tris HCl pH 7.5.
0.1 M ribose 5-phosphate in deionized water
deionized water

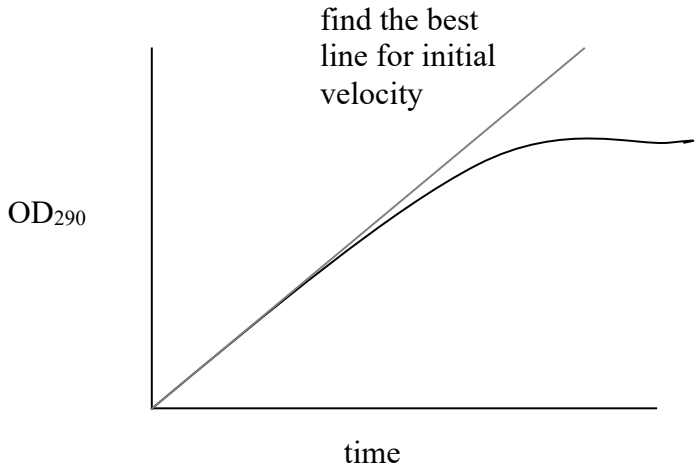
Protein stock solutions
0.04 mg/ml wild type RpiA in 50 mM Tris HCl pH 7.5
(You will be testing mutated proteins as well, but we don't know what concentration to prepare yet. When you make these stocks, be sure that they are in 50 mM Tris HCl pH 7.5, just like the wild type.)

Procedure for test with wild type enzyme:
All groups should start with this as a control, to make sure everything is working!

1. Mix:
50 µl of 1 M Tris HCl pH 7.5
100 µl of 0.1 M ribose 5-phosphate
(Here would be the place to add whatever else you want in the assay, e.g. more substrate / inhibitor / salts; adjust water volume as needed)
deionized water to 995 µl.
Use this to set the zero (blank) on the spectrophotometer.

2. Start the reaction by adding 5 µl of 0.04 mg/ml RpiA, then mix quickly (covering the cuvette with Parafilm, then tipping upside down once or twice to mix, is fastest, but be careful not to introduce any bubbles!), and put it back into the spectrophotometer.

3. Start measuring! Follow the change in absorbance with time (writing down the OD_{290} at 5 s intervals may be appropriate; it depends on how fast your reaction is going). Stop measuring when you are sure the initial (linear) phase is over, as in the picture. The experiment should look like this:



Once the line "flattens" out (at the right), you can stop measuring. It is that "initial rate" that you want to know.

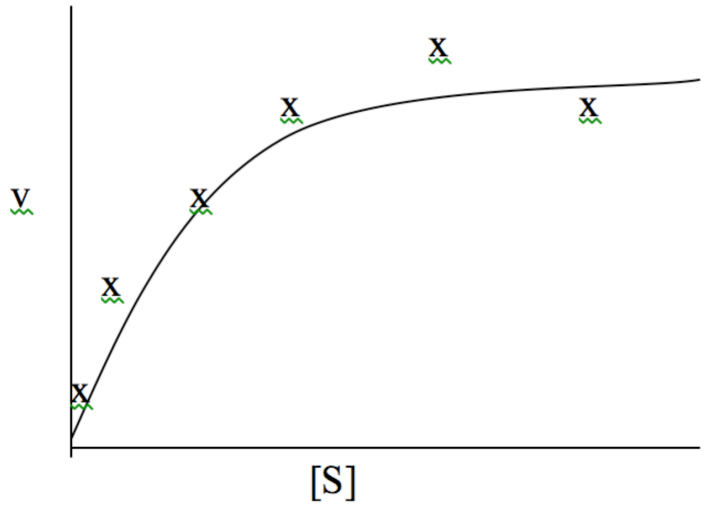
Data for wild-type spinach RpiA at 37 °C
K_m (for ribose 5-phosphate) = 1.3 mM
k_{cat} = 1500/s (Since we are measuring at room temperature, yours may be a bit slower.)
Molecular weight of spinach RpiA = 26 187 Da
(In the assay described above, [E] = 7.6 nM.)

Measuring k_{cat} and K_m for your mutants
Once you are sure that you have everything working for the wild type, start measuring for your mutants. Now that you know how the assays looks, decide how your team will work, giving everyone a useful job. For example, you might have one person "running" the spectrophotometer, putting samples in, and calling out the OD reading every 5 seconds. A second person might be writing down those values, while a third person is plotting the data on paper or computer (you will find it much easier to decide when the linear phase is over, if you look at this plot right away). The more efficiently your team works, the faster it will be to collect the information you need (and for us all to go home happy!).

Choose one of your mutants, and prepare a small amount of stock solution, just like you made for the wild type. The concentration of mutant RpiA needed will depend on the activity of the particular enzyme, but a good starting point is around 8 nM RpiA in the final reaction mixture. Then do a test just like the one you did for the wild type. Is the activity the same, higher, lower? If it is lower, think back to what you predicted for this mutation. Do you expect k_{cat} to be lower

(if so, add more enzyme next time). Do you expect K_m to be higher (if so, add more substrate next time). THINK!

You will want to create a Michaelis-Menten plot for each mutant enzyme, i.e. one that looks like the plot below. (For the moment, keep your plots in terms of change in absorbance per minute on the y-axis, and time on the x-axis. After all, that is what you see in the experiment, so it is easier to be sure you have done everything correctly.)



Make this plot as you go, to be sure that you have explored the full curve (if possible, [S] from about $K_m/10$ to K_m x 10). We want to see a plot like this for each of your mutants before you leave the lab.

For mutations that are designed to increase stability, you will need to do some tests at higher temperature as well, and compare to the wild type. Hopefully your unheated protein is just as active as the wild type.

Other calculations will be done later, just as you did in the kinetics assignment. For this, you will use

$A = \varepsilon \times l \times c$

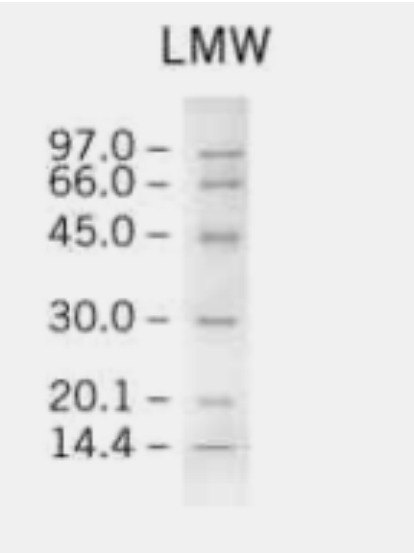
where l = path length, c = concentration of product, ε = extinction coefficient of product (i.e. = 72 $M^{-1}cm^{-1}$ for ribulose 5-phosphate)
to convert the measured change in absorbance per unit of time, to concentration ribulose 5-phosphate per unit of time, i.e. the rate of the reaction. Check that you have calculated k_{cat} correctly!

References:

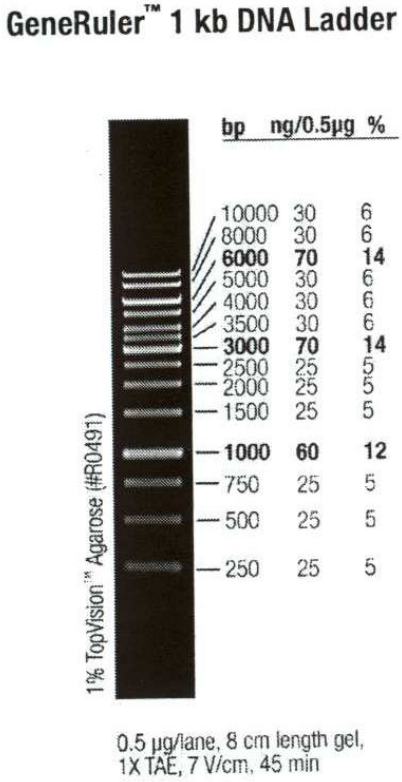
Sambrook J. and Russell D.W. (2001) Molecular Cloning Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Various manuals for kits and equipment will be available in the lab.

Appendix: Markers



A 3-µl aliquot of a two-fold dilution of LMW Marker Kit was separated on a 15% polyacrylamide gel.



Lysozyme 14.3 kDa RNase 13.7 kDa DNase 31 kDa RpiA 26187 Da

Important protein sizes

Presentation of lab results

Your talk should take about 15-20 minutes, which allows time for discussion of each group's results.

Please mail your presentation to Sherry ahead of time, but also bring it on a stick, if possible; it is impossible to have too many backup plans.

Here is the minimum of what you should include in your presentation:

What mutations you made, and the rationale for choosing them. Most people will need to make better pictures than they had for the presentation of the plan. Make sure you describe/show the structural setting of your mutation clearly, and state what you expect will happen to the protein's properties when you change to a new amino acid.

The results for the DNA-based experiments you did, including the gels (PCR mutagenesis, plasmid gel, analytical PCR, etc).

The results for the expression/purification experiments you did, including the gels (test expression PAGE, the final PAGE where you analyzed samples from the purified fractions, etc). Comment on any evidence for low expression, inclusion bodies, molecular weights that are odd, i.e. anything unusual! State the total yield of purified protein in terms of mg protein; compare to wild type value.

The results for the kinetics experiments you did, and any tests of stability. Show the kinetics plots (Michaelis-Menten, Lineweaver-Burke and Hanes-Woolf) for each of your mutants. It is probably best to present scatter plots for Michaelis-Menten, unless you actually have a curve that you fit. State the values for kcat and Km for each of your mutants. For "dead" mutants, show the data you do have, and where possible, place limits (upper or lower) on the kcat and Km values. For stabilization tests, show plots of absorbance changes for the trials you did.

Include anything else that the lab teachers asked you to include.

If you didn't get one or both of your mutations, or your protein didn't express/purify, just continue in the framework outlined above, using the wild type enzyme. As needed, include descriptions of procedures that differ from those in the lab manual.

Summarize your results, and give interpretations of the results you got. If things didn't work out, analyze as best you can, and try to figure out why. Did your mutants do what you expected them to do? Did you get your mutation, according to the sequencing results? To analyze your sequence data, you'll want a program. We suggest the following:
on a PC, http://www.technelysium.com.au/ChromasLite211Setup.exe
on a Mac, http://4peaks.en.softonic.com/mac

Provide thoughts for possible future work. What would you have done if you had more time?

Tips and things to remember:

Label all gels and other figures; walk through them, explaining what is happening step-by-step. Otherwise, your audience will have trouble following what you are doing. Don't expect them to figure everything out without your help!

Give units on all plots, and for all values reported.

Be sure you give molecular weights for markers (ladders), and other relevant information.

Keep your slides as simple and clear as possible, while still making your points. Titles and labels help the listener a lot, but don't give massive amounts of text.

Don't use dark letters or objects on black background, or light things on a light background. They disappear!

Use a reasonable number of significant figures. Report, for example, 6.4 mg/ml, not 6.4847262973 mg/ml.

Grading:
The criteria for grading the presentations are posted on the course web pages, so you can see them there.

Grading criteria for oral presentations of wet lab results

Hypothesis and Results sections are listed for the two mutations. Where a mutation was not obtained, analyze why it failed, and describe work that was done with the wild-type enzyme instead.

	Very good	*Good*	*Not so good*
Content/Knowledge			
Hypothesis, mutation 1	Hypothesis is correctly based on biochemical principles and several aspects have been analyzed. (2 pts)	Hypothesis is correctly based on biochemical principles, but reasoning is not clear. (1 pt)	Hypothesis is not correctly based on biochemical principles.
Hypothesis, mutation 2	Hypothesis is correctly based on biochemical principles and several aspects have been analyzed. (2 pts)	Hypothesis is correctly based on biochemical principles. (1 pt)	Hypothesis is not correctly based on biochemical principles.
DNA results, mutation 1	Clearly presents the results with own interpretations/explanations. (2 pts)	Correctly presents most of the results with some interpretation/explanations. (1 pt)	The results are incorrect or not clearly presented. No interpretations/explanations.
Expression/purification results, mutation 1	Clearly presents the results with own interpretations/explanations. (2 pts)	Correctly presents most of the results with some interpretation/explanations. (1 pt)	The results are incorrect or not clearly presented. No interpretations/explanations.
Kinetics results, mutation 1	Clearly presents the results with own interpretations/explanations. (2 pts)	Correctly presents most of the results with some interpretation/explanations. (1 pt)	The results are incorrect or not clearly presented. No interpretations/explanations.
DNA results, mutation 2	Clearly presents the results with own interpretations/explanations. (2 pts)	Correctly presents most of the results with some interpretation/explanations. (1 pt)	The results are incorrect or not clearly presented. No interpretations/explanations.
Expression/purification results, mutation 2	Clearly presents the results with own interpretations/explanations. (2 pts)	Correctly presents most of the results with some interpretation/explanations. (1 pt)	The results are incorrect or not clearly presented. No interpretations/explanations.
Kinetics results, mutation 2	Clearly presents the results with own interpretations/explanations. (2 pts)	Correctly presents most of the results with some interpretation/explanations. (1 pt)	The results are incorrect or not clearly presented. No interpretations/explanations.
Discussion	Analyzes and discusses the results based on own opinions. Say what you would do differently next time, or in further work (2 pts)	Shows understanding of the results. (1 pt)	Gives the impression of not fully understanding the results.
Presentation			
Performance	Correct language at an appropriate level. Speaks clearly and at a good pace. Delivery is fluent and expressive. (2 pts)	Mainly correct language. Usually speaks clearly. Delivery mainly fluent. (1 pt)	Frequently incorrect language. Only occasionally speaks clearly and at a good pace.
Technical aids	Technical aids significantly improve clarity of	Technical aids correctly support the presented topic. (1 pt)	No technical aids used or technical aids used that confuse audience

	presentation and add impact and interest. (2 pts)		
Composition	Contains introduction, results/discussion and a summary that are well-balanced relative each other. (2 pts)	Contains introduction, results/discussion and a summary, but balance could be better. (1 pt)	Lacks introduction, results/discussion or summary. Alternatively, there is a significant imbalance in the composition.