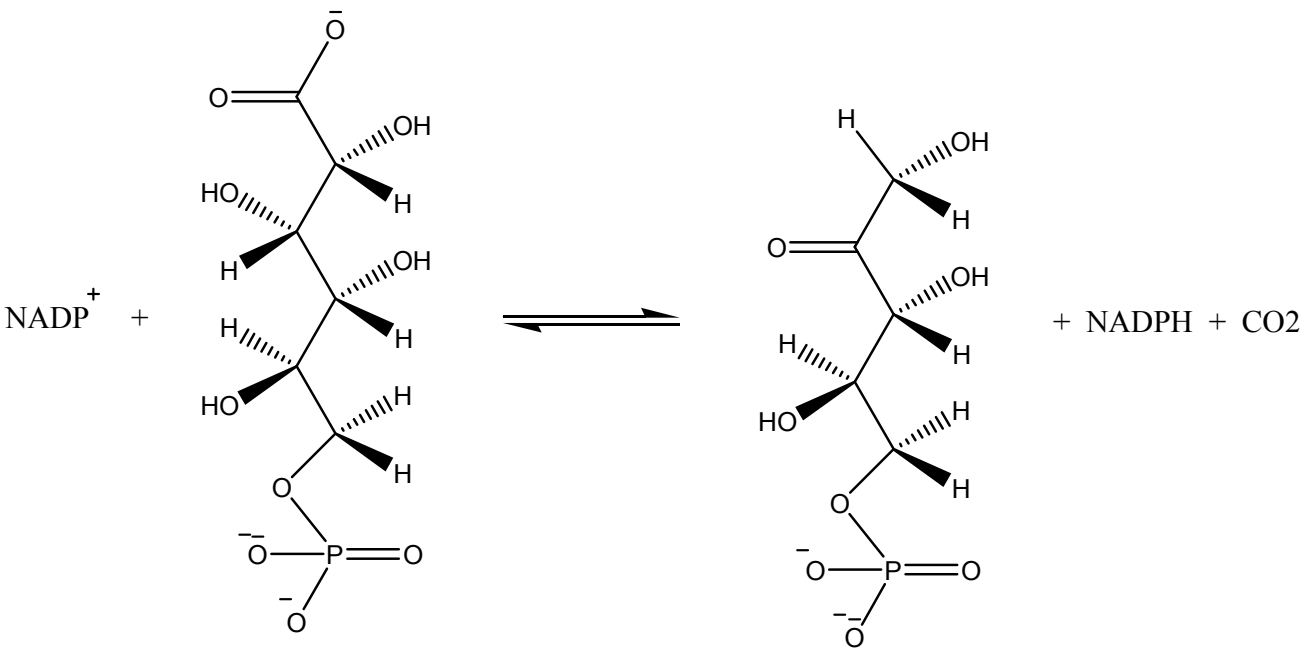
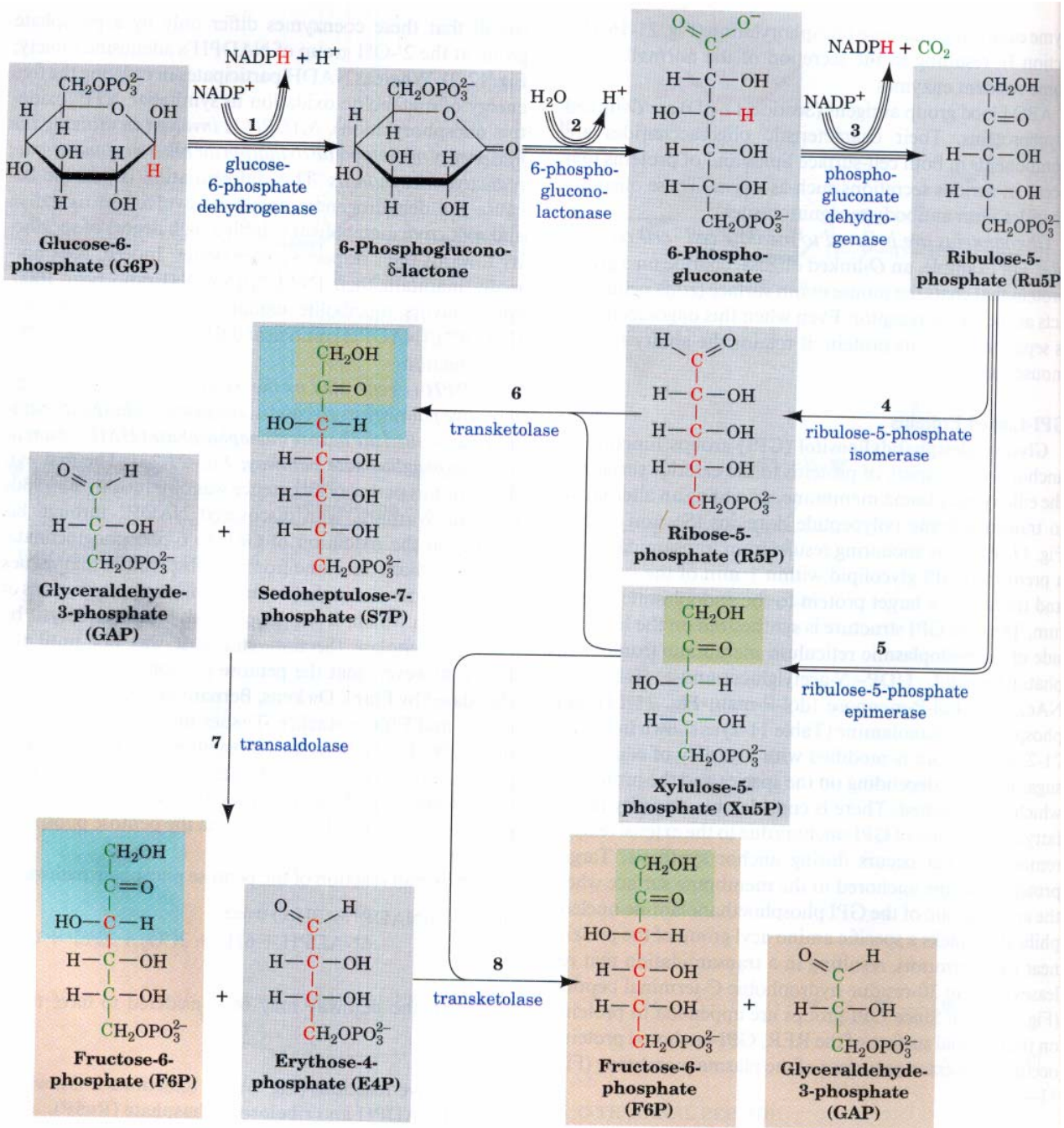
Substrate Binding in 6-Phosphogluconate Dehydrogenase (6-PGDH)

Introduction: 6-PGDH

- Part of the pentose phosphate pathway
- Catalyzes the reversible oxidative decarboxylation of 6-phosphogluconate (6PG) to ribulose 5-phosphate (R5P) and CO_2 under generation of NADPH

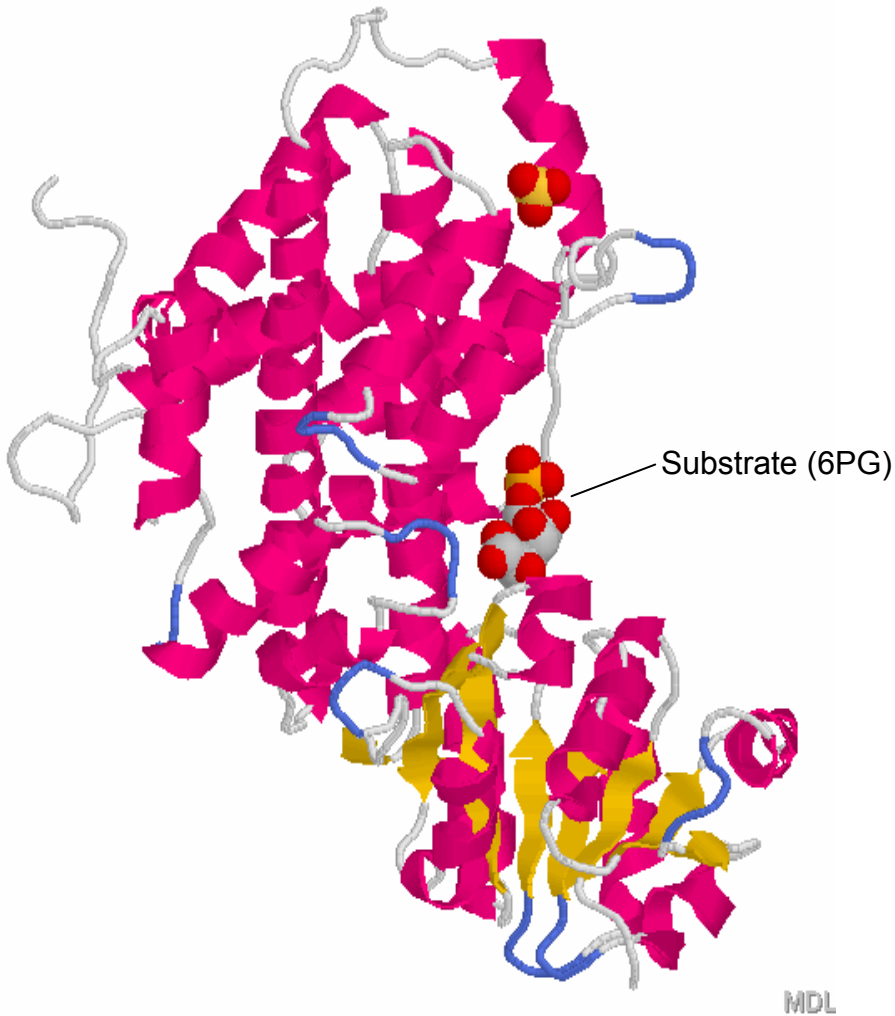


Pentose Phosphate Pathway



Structure: 6-PGDH

- Structure of apoenzyme and in presence of 6PG/ NADP; Ru5P/NADPH solved for sheep liver 6PGDH
- Dimeric protein
- Two identical Domains
- Both Domains involved in active site

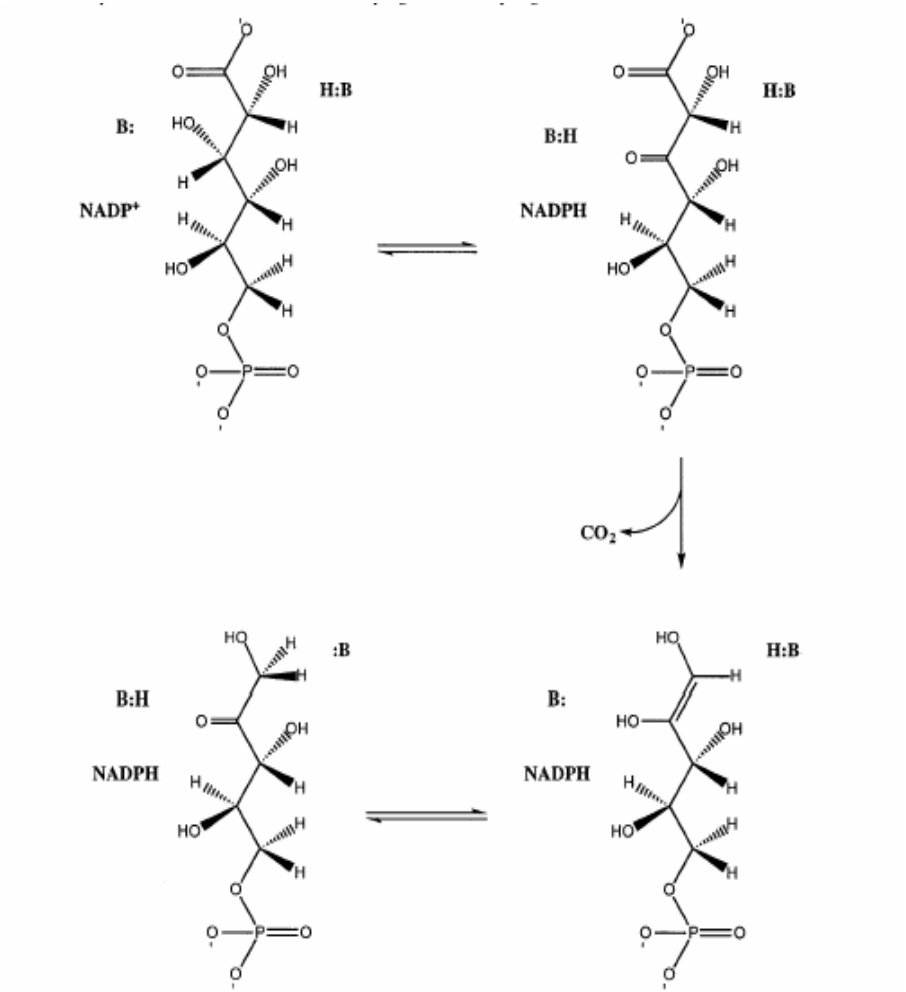


Introduction: 6-PGDH

- Chemical:
  General base - general acid mechanism

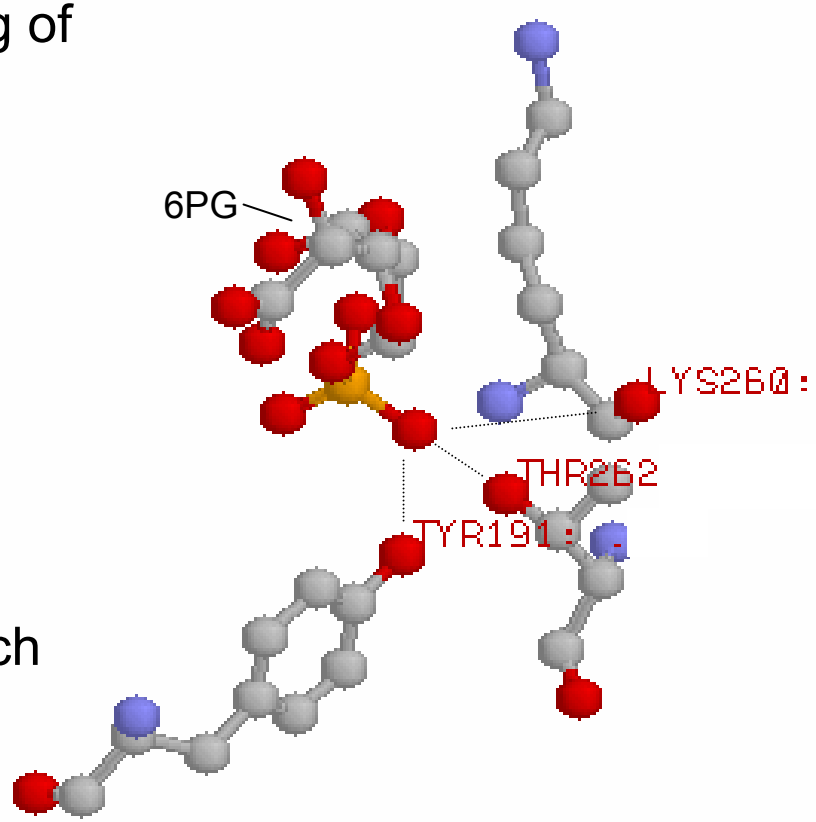
- K183 and N187 are in hydrogen-bonding distance from the 3-hydroyl of 6PG. K183 is most likely the general base

- E190 is in hydrogen-bonding distance from the C-1 of 6PG. 190 is most likely the general acid



Introduction: 6-PGDH

- Influence of each residue involved in assumed binding of the P-terminus (distance & energy)
- Residues:
  - Y191
  - T262
  - K260
  - R446 (2nd chain)
- Method: site-directed mutation of each of these residues to ALA



Data: Primer

Y191 [TAC ➔ GCC]

Sequence

5’ **G CAC AAC GGC ATA GAG TAC GGG GAC ATG CAG C** 3’

Primer 1 (FD1Y191A) [forward]

5’ **G CAC AAC GGC ATA GAG GCC GGG GAC ATG CAG C** 3’

Primer 2 (FD1Y191B) [reverse]

5’ **G CTG CAT GTC CCC GGC CTC TAT GCC GTT GTG C** 3’

Data: Primer

T262 [ACC ➔ GCC]

Sequence

5’ **GGG CAG AAG GGC ACC GGG AAG TGG ACC** 3’

Primer 1 (FD1T262A) [forward]

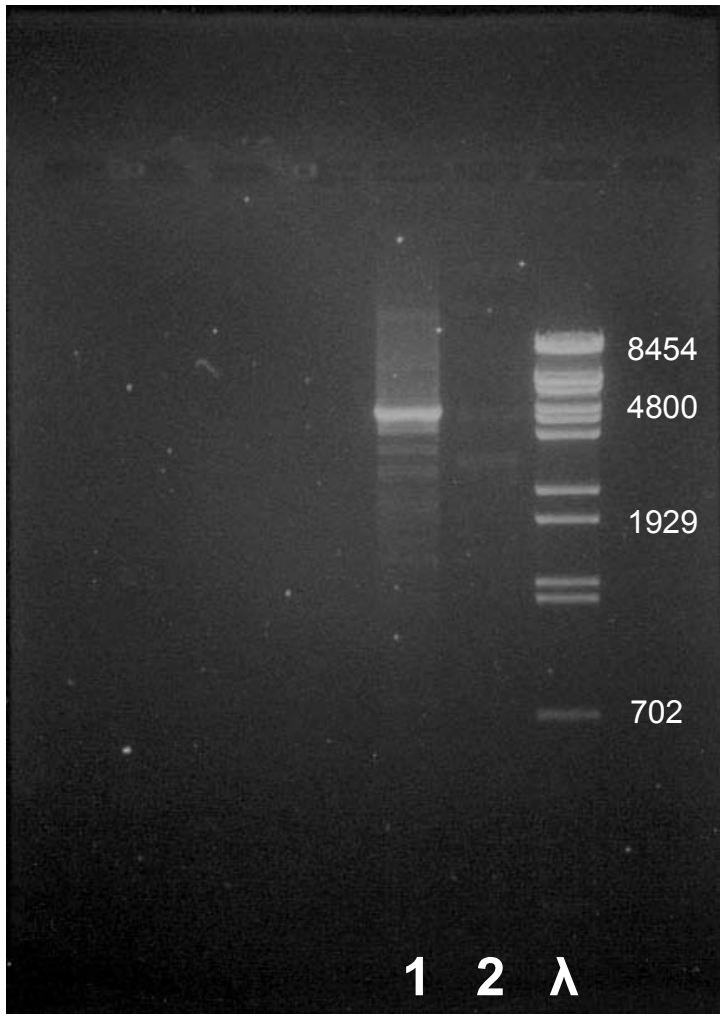
5’ **GGG CAG AAG GGC GCC GGG AAG TGG ACC** 3’

Primer 2 (FD1T262B) [reverse]

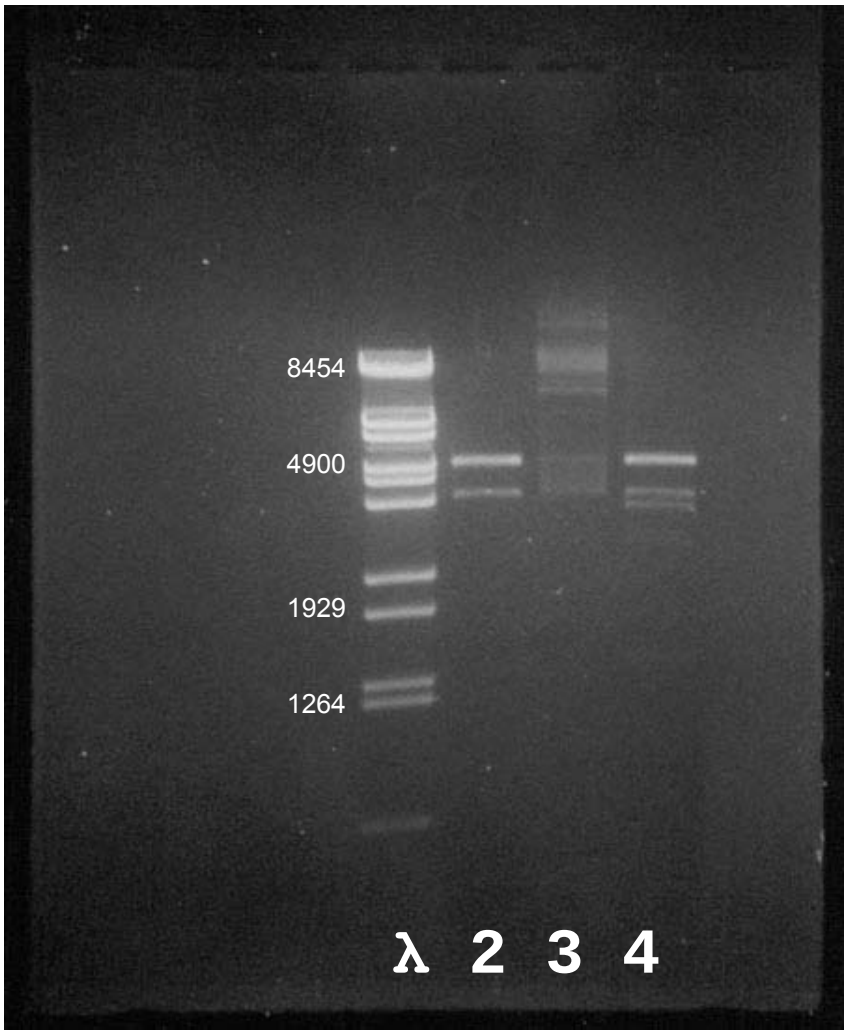
5’ **GGT CCA CTT CCC GGC GCC CTT CTG CCC** 3’

Data: Mutation

- PCRcontrol
- Lanes:
  1) T262A mutant
  2) Y191A mutant
  3) λDNA ladder
- Expected size of Plasmid: ~3.7kb

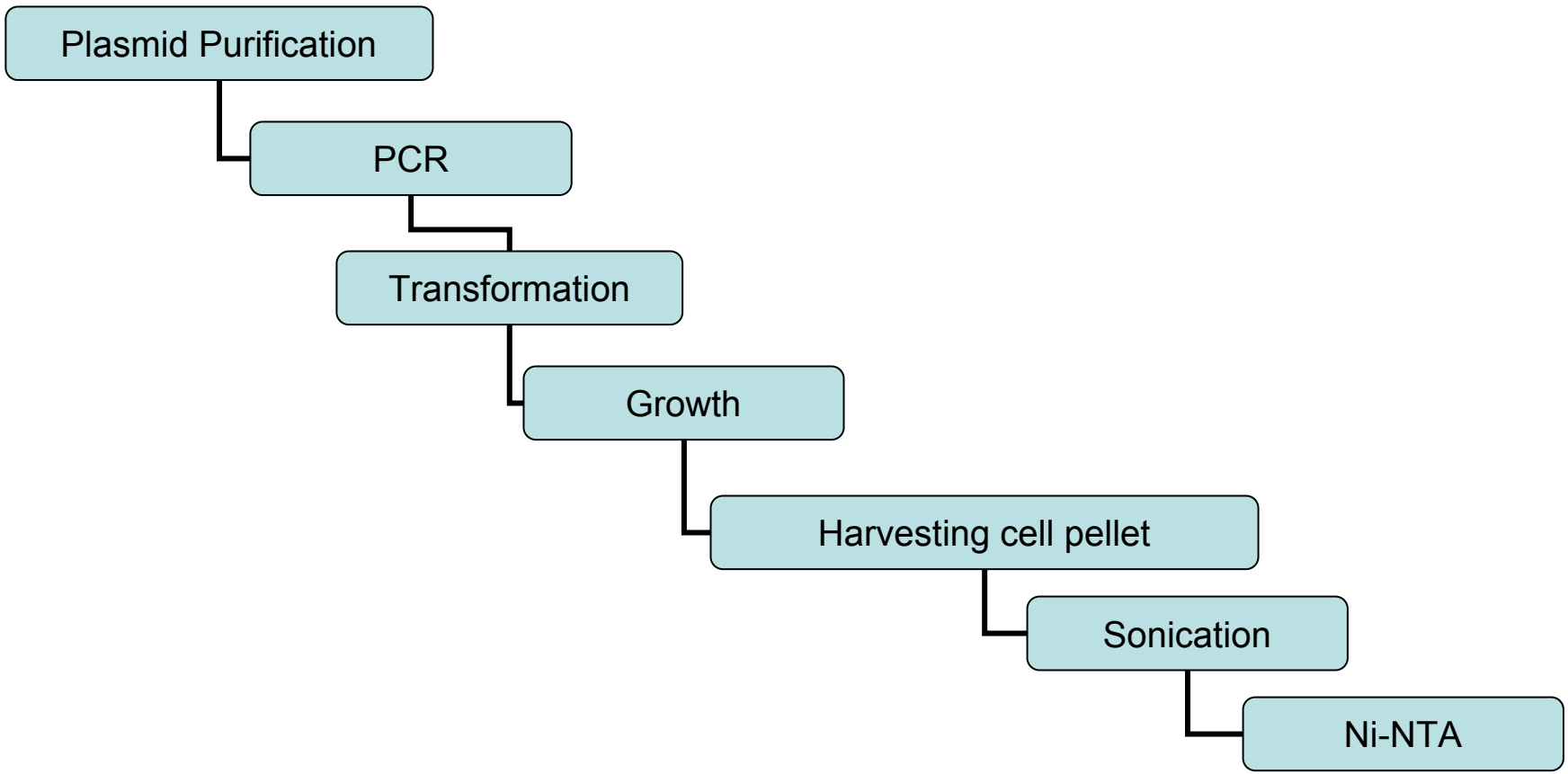


Data: Mutation



- Digest control (T262A)
- Sites:
  - Hind III
  - SpH I
- Lanes:
  1) λDNA ladder
  2) Hind III
  3) SpH I
  4) Both [1:1]
- Expected size of plasmid: 3.7+1.5=~5.2kb

Overview: Purification



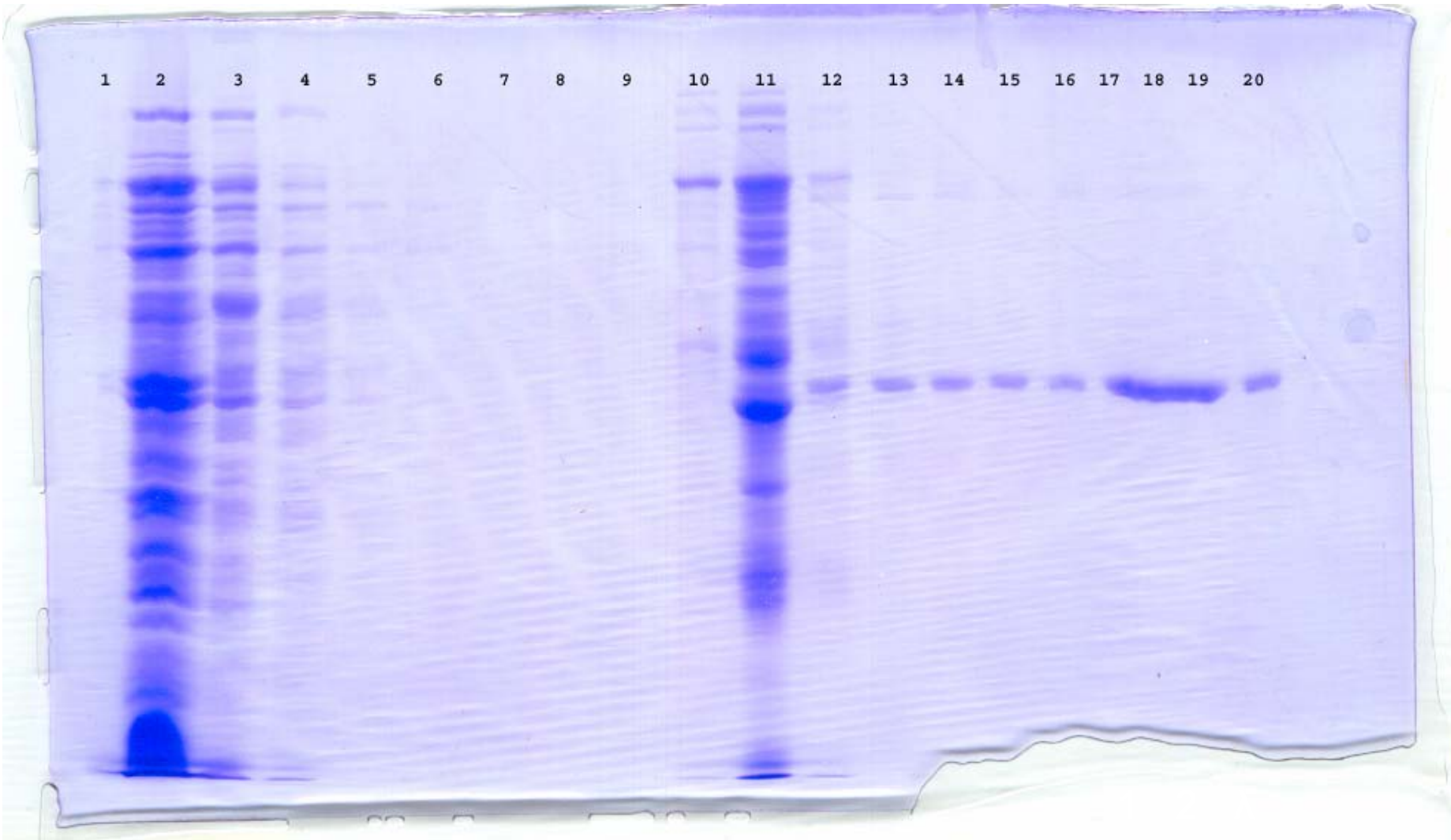
Protocol: Expression

- Bacterial strains growing @ 30°C in 1L of LB-broth w/ 100µg/ml ampicilin and 25 µg/ml kanamycin
- When A_{600} reaches 0.6 induce with IPTG to final concentration of 0.5 mM
- Growing for additional 5h @ 30°C
- Harvest by centrifugation at 4000rpm for 15min @ 4°C

Protocol: Purification

- Resuspend cell paste in 4 volumes of sonication buffer
  (50 mM sodium phosphate (pH 8.0);300 mM NaCl; 10 mM *β*-mercaptoethanol)
- Sonicate for 5x15sec on ice
- Centrifuge at 13,000g to pellet cell debris (20min)
- Bind supernatant to Ni-NTA resin, shake carefully for ~10min
- Pour into column
- Wash with sonication buffer (3-4 times)
- Elute with imidazol gradient (0.1; 0.2; 0.3; 0.4 M)
- Measure protein concentration for all fractions using Bradford essay, chose fractions corresponding to peak
- Precipitate enzyme by 75% ammonium sulfate, store @ 4°C
- Analyze purity via SDS-PAGE

Data: Mutation



Data: Purification

Lane	Fraction	Conc.	Lane	Fraction	Conc.
1	MW markers	-	11	Imidazol 0.1 c	High
2	Pellet	-	12	Imidazol 0.1 d	620
3	Wash 1	~ 1000	13	Imidazol 0.1 e	177
4	Wash 2	750	14	Imidazol 0.1 f	113
5	Wash 3	166	15	Imidazol 0.2 a	109
6	Wash 4	-	16	Imidazol 0.2 b	216
7	Wash 5	Low	17	**Imidazol 0.2 c**	**617**
8	Wash 6	Low	18	Imidazol 0.2 d	130
9	Imidazol 0.1 a	low	19	Imidazol 0.2 e	-
10	Imidazol 0.1 b	420	20	Imidazol 0.2 f	-

The End