

## References

- Adaskaveg J.E., and Hartin R. J.** (1997). Characterization of *Colletotrichum acutatum* isolates causing anthracnose of almond and peach in California. *Phytopathology* 87: 979- 987.
- Albersheim P. and Anderson A.** (1971). Host-pathogen interactions. III. Proteins from plant cell walls inhibit polygalacturonases secreted by plant pathogens. *Proceedings of the National Academy of Sciences of the United States of America* 68: 1815-1819.
- Andersen D. C. and Krummen L.** (2002). Recombinant protein expression for therapeutic applications. *Current Opinion in Biotechnology* 13: 117–123.
- Arendse M.S.** (1999). Molecular cloning and analysis of a polygalacturonase-inhibiting protein (PGIP) gene from apple. MSc Thesis. RAU University.
- Arendse M.S. Dubery I.A. and Berger D.K.** (1999). Isolation by PCR-based methods of a plant antifungal polygalacturonase inhibiting protein gene. *EJB*. 2(3): 152-159.
- Atkinson R.G., Schröder R., Hallett I.C., Cohen D., and MacRae E.A.** (2002). Overexpression of polygalacturonase in transgenic apple trees leads to a range of novel phenotypes involving changes in cell adhesion. *Plant Physiology* 129: 122-133.
- Barash I., Zilberman E., and Marcus L.** (1984). Purification of *Geotrichum candidum* endopolygalacturonase from culture and from host tissue by affinity chromatography on cross-linked polypectate. *Physiol. Plant. Path.* 25: 161-169.
- Bonnin E., Le Goff A., Körner R., Vigouroux J., Roepstorff P. and Thibault J.F.** (2002). Hydrolysis of pectins with different degrees and patterns of methylation by the endopolygalacturonase of *Fusarium moniliforme*. *Biochimica et Biophysica Acta* 1596 (2002): 83-94.
- Boudart G., Lafitte C., Barthe J.P., Frasez D. and Esquerré-Tugayé M.** (1998). Differential elicitation of defense responses by pectic fragments in bean seedlings. *Planta* 206: 86-94.
- Bradford M.M.** (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72: 248- 254.
- Bussink H.J.D., Kester H.C.M. and Visser J.** (1990). Molecular cloning, nucleotide sequence and expression of the gene encoding prepro-polygalacturonaseII of *Aspergillus niger*. *FEBS* 273 (1,2): 127 – 130.
- Bussink H.J.D., Buxton F.P., Fraaye B.A., de Graaff L.H. and Visser J.** (1992). The polygalacturonases of *Aspergillus niger* are encoded by a family of diverged genes. *Eur. J. Biochem.* 208: 83 – 90.

- Cabanne C., and Donèche B.** (2002). Purification and characterization of two isozymes of polygalacturonase from *Botrytis cinerea*. Effect of calcium ions on polygalacturonase activity. *Microbiol.Res.* 157: 1-7.
- Caprari C., Richter A., Bergman C., LoCicero S., Salvi G. and Cervone F.** (1993). Cloning and characterization of a gene encoding the endopolygalacturonase of *Fusarium moniliforme*. *Mycological Research* 97: 497- 505.
- Centis S., Dumas B., Fournier J., Marolda M. and Esquerrè-Tugayé, M.** (1996). Isolation and sequence analysis *Clpg1*, a gene coding for an endopolygalacturonase of the phytopathogenic fungus *Colletotrichum lindemuthianum*. *Gene* 170: 125-129.
- Centis S., Guillas I., Séjalon N., and Esquerrè-Tugayé, M.** (1997). Endopolygalacturonase genes from *Colletotrichum lindemuthianum*: cloning of *Clpg2* and comparison of its expression to that of *Clpg1* during saprophytic and parasitic growth of the fungus. *Molecular Plant-Microbe Interactions* 6: 769-775.
- Cereghino J.L. and Cregg J.M.** (1999). Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. *FEMS Microbiology Reviews* 24: 45-66.
- Chomczynski P. and Sacchi N.** (1987). *Anal Biochem.* 162: 156-159.
- Collmer A. and Keen N.T.** (1986). The role of pectic enzymes in plant pathogenesis. *Ann. Rev. Phytopathol.* 24: 383 – 409.
- Cooper R.M., Wardman P.A. and Skelton J.E.M.** (1981). The influence of cell walls from host and non-host plants on the production and activity of polygalacturonide-degrading enzymes from fungal pathogens. *Phys. Plant. Path.* 18: 239 – 255.
- Dal Degan F., Child R., Svendsen I. and Ulvskov P.** (2001). The cleavable N-terminal domain of plant endopolygalacturonases from clade B may be involved in a regulated secretion mechanism. *J.boil.chem.* 276: 35297 – 35304.
- De Lorenzo G., D'Ovidio R. and Cervone F.** (2001). The role of polygalacturonase-inhibiting proteins (PGIPs) in defense against pathogenic fungi. *Annu Rev. Phytopathol.* 39: 313- 35.
- De Lorenzo G. and Ferrari S.** (2002). Polygalacturonase-inhibiting proteins in defense against phytopathogenic fungi. *Current Opinion in Plant Biology* 5:295-299.
- D'Ovidio R., Mattei B., Roberti S. and Bellincampi D.** (2004). Polygalacturonases, polygalacturonase-inhibiting proteins and pectic oligomers in plant-pathogen interactions. *Biochimica et Biophysica Acta* 1696:237-244.
- Dugert M. and Ehrlich S.P.** (1974). Prolonged incubation in Calcium chloride improves competence of *Escherichia coli* cells. *Gene* 6:23-28 (Current protocols section 1.8.1).

**Dumas B., Centis S., Sarrazin N. and Esquerré-Tugayé, M.** (1999). Use of green fluorescent protein to detect expression of an endopolygalacturonase gene of *Colletotrichum lindemuthianum* during bean infection. *Appl. and Environ. Microbiol.* 65(4): 1769-1771.

**Esquerré-Tugayé M., Boudart G. and Dumas B.** (2000). Cell wall degrading enzymes, inhibitory proteins, and oligosaccharides participate in the molecular dialogue between plants and pathogens. *Plant Physiol. Biochem.* 38 (1/2): 157-163.

**Evans, G. I., Lewis, G. K., Ramsay, G., and Bishop, V. M.** (1985). Isolation of Monoclonal Antibodies Specific for c-myc Proto-oncogene Product. *Mol. Cell. Biol.* 5: 3610-3616.

**Federici L., Caprari C., Mattei B., Savino C., Di Matteo A., De Lorenzo G., Cervone F. and Tsernoglou D.** (2001). Structural requirements of endopolygalacturonase for the interaction with PGIP (polygalacturonase inhibiting protein). *PNAS.* 98 (23): 13425–13430.

**Glass N. L. and Donaldson G. C.** (1995). Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Appl. Environ. Microbiol.* Vol 61 (4): 1323-1330.

**Gognies S. and Belarbi A.** (2002). Endopolygalacturonase of *Saccharomyces cerevisiae*: involvement in pseudohyphae development of haploids and in pathogenicity on *Vitis Vinifera*. *Plant Science.* 163: 759 – 769.

**Gonzalez-Candelas G.W.L., and Kolattukudy P.E.** (1995) Cloning of a novel constitutively expressed pectate lyase gene *pelB* from *Fusarium solani* f. sp. *pisi* (*Nectria haematococca*, mating type VI) and characterization of the gene product expressed in *Pichia pastoris*. *J. Bacteriol.* 177: 7070-7077.

**Gonzalez-Candelas G. W.L., and Kolattukudy P.E.** (1996). Cloning of a novel *pelD* gene expressed uniquely in planta by *Fusarium solani* f. sp. *pisi* (*Nectria haematococca*, mating type VI) and characterization of its protein product as an endopectate lyase. *Arch. Biochem. Biophys.* 332: 305-312.

**Gurr S. J., Unkles S. E. and Kinghorn J. R.** (1987). In *Gene Structure in Eukaryotic Microbes* (ed. J.R. Kinghorn), pp. 93-139. IRL Press: Oxford, U.K.

**Houard S., Heinderyckx M. and Bollen A.** (2002). Engineering of non-conventional yeasts for efficient synthesis of macromolecules: the methylotrophic genera. *Biochimie.* 84: 1089–1093.

**Isshiki A., Akimitsu K., Yamamoto M. and Yamamoto H.** (2002). Endopolygalacturonase is Essential for citrus black rot caused by *Alternaria citri* but not brown spot caused by *Alternaria alternata*. *MPMI.* 14: 749–757.

**Johnston D.J. and Williamson B.** (1992). Purification and characterisation of four polygalacturonases from *Botrytis cinerea*. *Mycol. Res.* 5: 343-349.

**Koch S.H.** (1996). *Colletotrichum* spp. on dry beans and lupins in South Africa. PhD. thesis. University of Pretoria, South Africa.

**Koch S.H., Ghebremariam D.S. and Swart W.J.** (2002). Susceptibility of lupin cultivars to South African isolates of *Colletotrichum gloeosporioides* associated with lupin anthracnose. *African Plant Protection* 8 (1 &2).

**Lafitte C., Barthe J.P., Gansel X., Dechamp-Guillaume G., Faucher C., Mazau D. and Esquerré-Tugayé M.T.** (1993). Differential induction by endopolygalacturonase of  $\beta$ -1,3-glucanases in *Phaseolus vulgaris* isoline susceptible and resistant to *Colletotrichum lindemuthianum* race  $\beta$ . *MPMI*. 6: 628-634.

**Li J. and Goodwin P.H.** (2002). Expression of *cmpg2*, an endopolygalacturonase gene of *Colletotrichum gloeosporioides* f.sp. *malvae*, in culture and during infection of *Malva pusilla*. *J. Phytopathology*. 150: 213- 219.

**Markovič O. and Janeček Š.** (2001). Pectin degrading glycoside hydrolases of family 28: sequence-structural features, specificities and evolution. *Protein Engineering*. 14: 615-631.

**Nakamura M., Suprapta DN., Iwai H. and Arai K.** (2001). Comparison of endopolygalacturonase activities of citrus and non-citrus races of *Geotrichum candidum*, and cloning and expression of the corresponding genes. *Molecular Plant Path.* 2: 265-274.

**Nirenberg H.I., Feiler U. and Hagedorn G.** Description of *Colletotrichum lupini* comb. nov. in modern terms. (2002). *Mycologia* 94 (2): 307-320.

**Oelofse D.** (2003). Molecular strategies towards anthracnose resistance in lupin. PhD thesis. University of Pretoria, South Africa.

**Oeser B., Heidrich P.M., Muller U., Tudzynski P. and Ten Berge K.B.** (2002). Polygalacturonase is a pathogenicity factor in the *Claviceps purpurea*/rye interaction. *Fungal Genetics and Biology*. 36: 176-186.

**Pařenicová L., Benen J.A.E., Kester H.C.M. and Visser J.** (2000 a). PgaA and pgaB encode two constitutively expressed endopolygalacturonases of *Aspergillus niger*. *Biochem. J.* 345: 637-644.

**Pařenicová L., Kester H.C.M., Benen J.A.E. and Visser J.** (2000 b). Characterisation of a novel endopolygalacturonases from *Aspergillus niger* with unique kinetic properties. *FEBS Letters*. 467 (2-3): 333-336.

**Poinssot B., Vandelle E., Bentéjac M., Adrian M., Levis C., Brygoo Y., Garin J., Sicillia F., Coutos- Thévenot P. and Pugin A.** (2003). The endopolygalacturonase 1 from *Botrytis cinerea* activates grapevine defense reactions unrelated to its enzymatic activity. *MPMI* 16 (6): 553-564.

**Raeder U. and Broda P.** (1985). Rapid preparation of DNA from filamentous fungi. *Lett Appl. Microbiol.* 1:17-20.

- Rai M. and Harish P.** (2001). Expression systems for production of heterologous proteins. *Curr. Science*. 80 (9): 1121- 1128.
- Rayner R.W.**, A mycological colour chart. (1970). Commonwealth Mycological Institute and British Mycological Society: Kew, England.
- Ridley B.L., O'Neill M.A. and Mohnen D.** (2001). Pectins: structure, biosynthesis, and oligogalacturonide-related signaling, *Phytochemistry*.57: 929–967.
- Saitou N. and Nei M.** (1978). The neighbour joining method: a new method for reconstructing phylogenetic tree. *Molecular Biology and Evolution* 4: 406- 425.
- Sambrook J., Fritsch E.F., and Maniatis T.** (1989). *Molecular Cloning. A laboratory manual* (2<sup>nd</sup> edition). Cold Spring Harbour Laboratory Press, Cold Spring Harbour, NY, USA.
- Scott- Craig J.S., Cheng Y. Cervone F., De Lorenzo G., Pitkin J.W. and Walton J.D.** (1998). Targeted Mutants of *Cochliobolus carbonum* Lacking the Two Major Extracellular Polygalacturonases. *Appl. Environ. Microbiol.* 64 (4): 1497–1503.
- Scott- Craig J.S., Panaccione D. G., Cervone F. and J. D. Walton.** (1990). Endopolygalacturonase is not required for pathogenicity of *Cochliobolus carbonum* on maize. *Plant Cell* 2: 1191–1200.
- Seideman C. E., Struhl K., Sheen J., Jessen T.** (1997). *Current protocols in molecular biology*. Section 1.8.1.
- Shieh M., Brown R. L., Whitehead M. P., Cary J. W., Cotty P.J., Cleveland T. E. and Deani R. A.** (1997). Molecular Genetic Evidence for the Involvement of a Specific Polygalacturonase, P2c, in the Invasion and Spread of *Aspergillus flavus* in Cotton Bolls. *Appl. Environ. Microbiol.* 63: 3548 – 3552.
- Sreenivasaprasad S., Mills P. R. and Brown A. E.** (1994). Nucleotide sequence of the rDNA spacer 1 enables identification of isolates of *Colletotrichum* as *C. acutatum*. *Mycological Research*. 98: 186-188.
- Swofford D.L.** (1999). Paup\*. Phylogenetic analysis using parsimony (\*and other methods), version 4. Massachusetts: Sinauer Associates, Sunderland.
- Talhinhas P., Sreenivasaprasad S., Neves-Martins J., and Oliveira H.** (2002). Genetic and morphological characterization of *Colletotrichum acutatum* causing anthracnose of lupins. *Phytopathology*. 92: 986-996.
- Taylor R.J. and Secor G.A.** (1988). An improved diffusion assay for quantifying the polygalacturonase content of *Erwinia* culture filtrates. *Phytopathology*. 78 (8): 1101-1103.
- Ten Have A., Mulder W., Visser J. and van Kan J.A.L.** (1998). The endopolygalacturonase gene Bcpg1 is required for full virulence of *Botrytis cinerea*. *Molecular Plant Microbe Interactions*. 11: 1009-1016.

**Ten Have W. A., van Kan J.A.L. and Visser J.** (1999). Cloning and partial characterization of endopolygalacturonase genes from *Botrytis cinerea*. Appl. Environ. Microbiol. 65 (4): 1596 – 1602.

**Ten Have A.**, PhD Thesis. (2000) Wageningen University. The Netherlands.

**The Arabidopsis Genome Initiative.** (2002). Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature 408: 796 –815.

**Torki M., Mandaron P., Mache R. and Falconet D.** (2000). Characterization of a ubiquitous expressed gene family encoding polygalacturonase in *Arabidopsis thaliana* Gene 242: 427–436.

**Van Santen Y., Benen J.A. E., Schröter K., Kalk K.H., Armand S., Visser J., Bauke W. D.** (1999). 1.68-Å Crystal Structure of Endopolygalacturonase II from *Aspergillus niger* and Identification of Active Site Residues by Site-directed Mutagenesis. J. Biol. Chemistry 274 (43): 30474–30480.

**Van Wyk M., Roux J., Barnes I., Wingfield B., Liew E., Assa B., Summerell B. and Wingfield M.** (2004). *Ceratocystis polychroma* sp. nov., a new species from *Syzygium aromaticum* in Sulawesi. Studies in Mycology 50: 273- 282.

**Warrens A. N., Jones M. D., and Lechler R.I.** (1997). Splicing by overlap extension by PCR using asymmetric amplification: an improved technique for the generation of hybrid proteins of immunological interest. Gene 186: 29 –35.

**Whitehead M.P., Shieh M.T., Cleveland T.E., Cary J.W. and Dean R.A.** (1995). Isolation and characterisation of polygalacturonase genes (*pecA* and *pecB*) from *Apergillus flavus*. Appl. Environ. Microbiol. 61(9): 3316-3322.**Wubben J.P., Mulder**

**Yakoby N., Kobiler I., Dinoor A. and Prusky D.** (1999). pH regulation of pectate lyase secretion modulates the attack of *Colletotrichum gloeosporioides* on avocado fruits. Appl. Environ. Microbiol. 66 (3): 1026-1030.

**Yang H.A. and Sweetingham M.W.** (1998). The taxonomy of *Colletotrichum* isolates associated with lupin anthracnose. Aust. J. Agric.Res. 49: 1213-23.

## Appendix A

### TE- buffer

10 mM Tris- HCl  
1 mM EDTA  
pH 8.0

### Genomic DNA extraction buffer

200mM Tris HCl pH8.5  
250 mM NaCl  
25mM EDTA  
0.5 % SDS

### 2 × Wash buffer

2 × SSC  
0.1% SDS

### 0.5 × Wash buffer

0.5 × SSC  
0.1% SDS

### Maleic acid buffer (Southern blot)

0.1 M Maleic acid  
0.15 M NaCl  
Adjust pH to 7.5 with NaOH (solid)

### Washing Buffer (Southern blot)

0.1 M Maleic acid  
0.15 M NaCl  
Adjust pH to 7.5 with NaOH (solid)  
0.3 % (v/v) Tween 20

### Detection buffer (Southern blot)

0.1 M Tris-HCl  
0.15 M NaCl  
pH 9.5

**Formamide hybridisation buffer (Southern blot)**

5 × SSC  
50 % formamide, deionised  
0.1 % sodium-lauroylsarcosine  
0.02 % SDS  
2% Blocking agent (fat-free milk powder)

**Pectin medium**

NH <sub>4</sub> NO <sub>3</sub>	1g / 500ml
KH <sub>2</sub> PO <sub>4</sub>	0.5g / 500ml
MgSO <sub>4</sub>	0.05g / 500ml
Yeast extract	0.25g / 500ml
NaOH	0.5g / 500ml
D-maleic acid	1.5g / 500ml
0.25 g/ 25 ml washed pectin	

**BMMY (Buffered methanol-complex medium)**

1% yeast extract  
2% peptone  
100mM potassium phosphate, pH 6.0  
1.34 % YNB  
4 × 10<sup>-5</sup> % biotin  
1% glycerol or 0.5% methanol

**LSLB (Low Salt Luria Broth) Medium**

10 g Tryptone  
5 g NaCl  
5 g Yeast Extract

Combine the dry reagents above and add deionized, distilled water to 950 ml.  
Adjust pH to 7.5 with 1 N NaOH. Bring the volume up to 1 liter. For plates, add 15 g/L agar before autoclaving.  
Autoclave on liquid cycle at 15 lbs/sq. in. and 121°C for 20 minutes.  
Allow the medium to cool to at least 55°C before adding the Zeocin to 25 µg/ml final concentration.  
Store plates at 4°C in the dark. Plates containing Zeocin. are stable for 1-2 weeks.

**5× RNA Loading buffer**

Saturated bromophenol blue	16µl
500 mM EDTA, pH 8.0	80µl
37% (12.3 M) formaldehyde	700µl
100% glycerol	2ml
Formamide	3.084ml
10×FA gel buffer	4ml
RNase-free water to 10 ml	

**10× FA gel buffer**

200mM 3-[Morpholino]propanesulfonic acid(MOPS) (free acid)  
50mM Sodium acetate  
10 mM EDTA  
pH to 7.0 with NaOH

**1× FA gel buffer**

10 × FA gel buffer	100 ml
37% (12.3 M)	20 ml
RNase-free water to 1L	

**SDS-PAGE separating gel (10 %)**

Acrlamide stock (30%)	1650 µl
Tris- HCl (1.5 M) pH 6.8	620 µl
SDS (10%)	50 µl
APS	25 µl
Temed	8 µl
dH <sub>2</sub> O	2020 µl

**SDS-PAGE stacking gel**

Tris-HCl (0.5M) pH 6.8	620 µl
SDS (10%)	25 µl
Acrlamide stock (30%)	330 µl
APS (10%)	15 µl
Temed	8 µl
dH <sub>2</sub> O	1520 µl

**Loading Buffer (SDS-PAGE) 3 ×**

Tris-HCl pH 6.8 (50mM)	3 ml
Glycerol (8%)	2.4 ml
SDS (20%)	2,4 ml
2-β-mercaptoetanol (4%)	1.2 ml
dH <sub>2</sub> O	to an end volume of 10 ml
Bromophenolblue	to the desired colour intensity

**Transfer stock solution 10 × (Western Blot)**

Tris (base)	96.8 g/l
Glycine	9.74 g/l
pH	9.2

**Transfer Buffer (Western Blot)**

Methanol	200 ml
Transfer stock solution 10×	100 ml
dH <sub>2</sub> O	700 ml

**PBS 10 × Stock**

NaCl	1.3 M
Na <sub>2</sub> HPO <sub>4</sub> · 12H <sub>2</sub> O	70 mM
NaH <sub>2</sub> PO <sub>4</sub> · 1H <sub>2</sub> O	30 mM
pH	7.3

**Blocking buffer (Western blot)**

1× PBS	
Tween	(0.2%)
Fat free milk powder	(5%)
or BSA	(3%)

**Washing buffer (Western blot)**

1× PBS	
Tween	(0.2%)
H <sub>2</sub> O (ultra pure)	

**Incubation buffer (Western blot)**

1× PBS	
Tween	(0.2%)
BSA	(0.5%)
H <sub>2</sub> O (ultra pure)	

**1 × TNE Buffer**

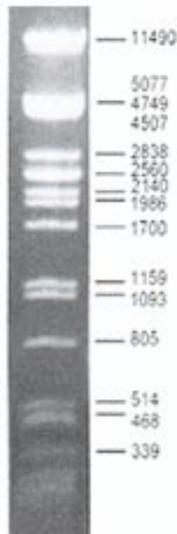
10 mM Tris-HCl pH 8.0

1 mM EDTA pH 8.0

0.2 M NaCl

pH 7.4

## Appendix B



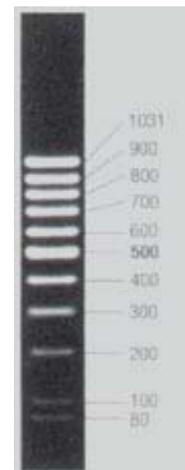
**$\lambda$  *Pst*I generated DNA molecular marker marker**



**1KB plus DNA molecular marker**

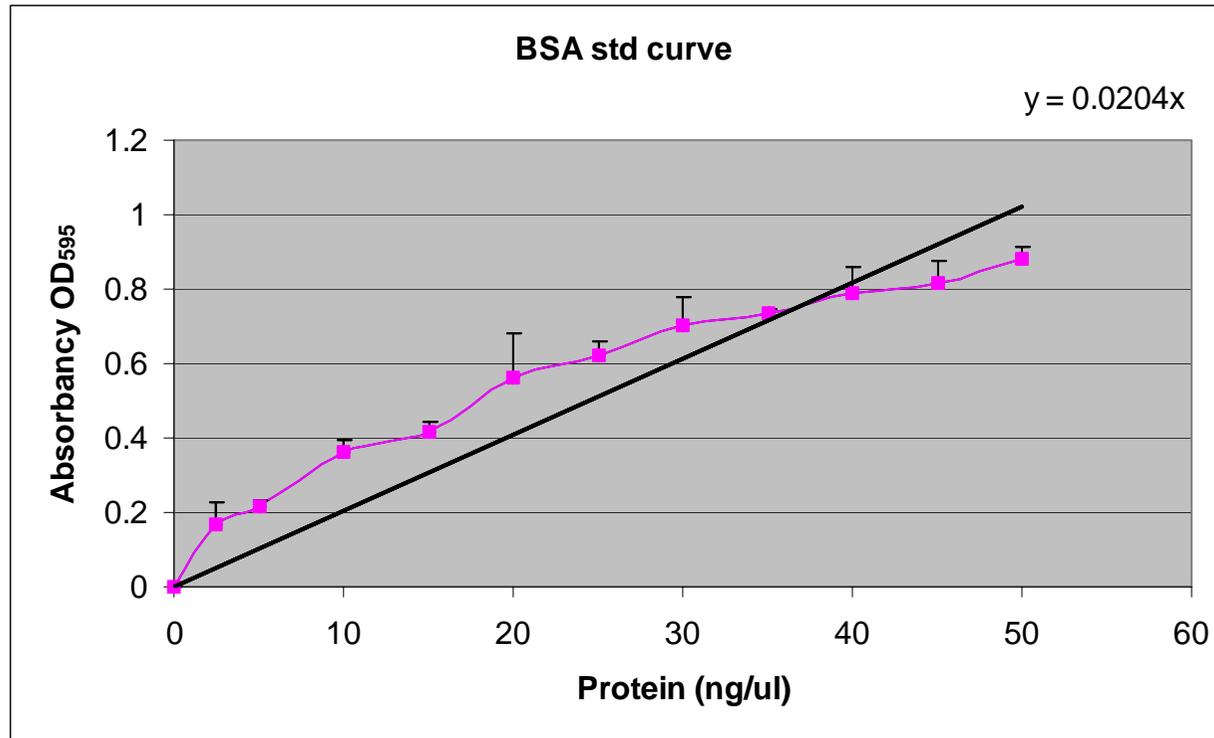


**SDS protein markers**



**100 bp molecular marker**

## Appendix C



**BSA standard curve for the Biorad microassay procedure performed in triplicate.**



















