Diagnostics
Diagnostic

Grapevine flavescence dorée phytoplasma

Specific scope

This standard describes a diagnostic protocol for Grapevine flavescence dorée phytoplasma.

Specific approval and amendment

Approved in 2005-09.

Introduction

Grapevine flavescence dorée phytoplasma (FD) belongs to the elm yellows group (16 SrV). It is one of a complex of disorders of *Vitis vinifera*, known as grapevine yellows, associated with the presence of phytoplasmas (Caudwell *et al*., 1971). Though characterized by similar symptomatology, these disorders (most of which are localized in distribution) are caused by different phytoplasmas belonging to six out of the twenty subclades so far distinguished on the basis of molecular evidence (Table 1) (Boudon-Padieu, 2003, 2005). The other yellows diseases of grapevine occurring in Europe (Table 1) are widespread in other hosts, and are not considered to be quarantine pests. Spread of flavescence dorée occurs through infected grapevine planting material and through its vector, the cicadellid *Scaphoideus titanus*.

Identity

Name: Grapevine Flavescence dorée phytoplasma
Taxonomic position: *Bacteria*, *Firmicutes*, *Mollicutes*, *Acholeplasmatales*, *Acholeplasmataceae*
Provisional taxon: Phytoplasma Elm Yellows (EY) Group or16SrV
Phytosanitary categorization: EPPO A2 list N° 94; EU Annex designation II/A2.

Detection

Disease symptoms

Flavescence dorée can be recognized in the field by the following symptoms, which develop mainly in summer (July onwards). Leaves turn yellow or red depending on the cultivar. They roll downward and become brittle (Figs 1–3). The interveinal areas of leaves may become necrotic. Shoots show incomplete lignification and rows of black pustules develop on the green bark along the diseased branches; they are thin, rubbery and hang pendulously. During winter they blacken and die. The inflorescences dry out and fall off. Fruit setting is reduced. In later infections, bunches are irregular and berries become shrivelled. They have a significantly lower sugar content and higher acidity compared to healthy grapes. Flavescence dorée occurs randomly in vineyards (this is perhaps associated with vine-to-vine transmission by the vector). It may occur in rootstocks, but without conspicuous symptoms. Most grapevine cultivars are affected by flavescence dorée. Cv 'Chardonnay' is particularly susceptible and sensitive to all grapevine yellows, especially flavescence dorée.

Fig. 1 Leaves of grapevine cv. Chardonnay affected by flavescence dorée showing typical yellowing of the laminar blade and rolling of margins. Leaves present a triangular shape [photograph courtesy of Dr. Federico Bondaz, Plant Protection Unit of Val d'Aosta region (IT)].

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Table 1 Current status of molecular characterization, biology and vectors of phytoplasmas causing grapevine yellows diseases (Boudon-Padieu, 2003, 2005)

Grapevine yellows disease	Phytoplasma name	Ribosomal group (subgroup)	Known insect vector to grapevine	Preferred host plants of vector	Occurrence
Flavescence dorée	FD	16SrV (-C, -D) or EY	*Scaphoideus titanus* Ball	*Vitis* sp.	France, Italy, Spain, Serbia, Slovenia, Switzerland
Palatinate grapevine yellows	PGY	16SrV or EY	*Oncopsis alni* Schrank	*Alnus glutinosa* L.	Germany
Bois noir, Legno nero, Vergilbungskrankheit Schwarzholzkrankheit	stolbur	16SrXII-A or stolbur	*Hyalesthes obsoletus* Sign	*Convolvulus arvensis* L. *Urtica dioica* L., *Ranunculus*, *Solanum*, *Lavandula*	Europe, Israel, Lebanon
Australian grapevine yellows	*Candidatus* Phytoplasma australiense	16SrXII-B	ND	ND	Australia
Australian grapevine yellows	*Candidatus* Phytoplasma australasia	16SrII FBP	ND	ND	Australia
Buckland valley grapevine yellows (Aus) grapevine yellows	BVGY	16SrI-related or AY-	ND	ND	Australia
	Aster yellows	16SrI (-B, -C) or AY	ND	ND	Italy, Chile
North American grapevine yellows (NAGY)	Virginia grapevine yellows I (VGY I)	16SrI-A or AY	ND	ND	Virginia (UA)
	Western X Virginia grapevine yellows III (VGYII)I	16SrIII-I or WX	ND	ND	New York (US) Virginia (US)

Fig. 2 Leaves of grapevine cv. Bellone affected by stolbur phytoplasmas. Symptoms are indistinguishable from the ones observed in grapevine affected by flavescence dorée. To perform molecular diagnosis is the only way to discriminate phytoplasmas belonging to different groups (Photograph courtesy of Dr. Federico Bondaz, Plant Protection Unit of Val d'Aosta region (IT)).

Fig. 3 Leaves of red grapevine cultivars affected by flavescence dorée showing rolling and reddening of the laminae. Symptoms are often present on a single shoot of the plant (Photograph courtesy of Dr. Federico Bondaz, Plant Protection Unit of Val d'Aosta region (IT)).

Identification of flavescence dorée *sensu stricto*

Biological assay

The biological assay proposed by OEPP/EPPO (1994) is not suitable for reliable or specific detection or identification of flavescence dorée, and is no longer recommended.

Serological assay

ELISA with polyclonal and monoclonal antibodies have been used for detection of flavescence dorée in vector

(Boudon-Padieu *et al*., 1989) and grapevine (Caudwell & Kuszala, 1992; Kuszala *et al*., 1993; Kuszala, 1996), but the method relies on availability of antibodies which are not sold commercially. It has been replaced in practice by PCR, which is versatile, specific and sensitive.

Molecular methods

Two methods can be used, the first one with two variants. In the first method, direct PCR with generic primers for phytoplasma 16S rDNA amplification is followed by nested PCR using group-specific primers (a), or by nested PCR using a second generic primer pair (b). In this latter case, the amplification product can be submitted to RFLP analysis for identification of the causal phytoplasma. The second method is a multiplex nested-PCR assay that allows direct identification of phytoplasmas in the elm yellows (16SrV) (Flavescence dorée) and stolbur (16SrXII) (Bois noir) groups, the two main grapevine yellows present in Europe (c).

These methods are described in Appendix 1.

Reference material

Institut national de la recherche agronomique, Equipe phytoplasmes, UMR PME, INRA, BP 86510, 21065 DIJON Cedex, France.

Institut national de la recherche agronomique, UMR GDPP Bordeaux, BP 81, 33883 VILLENAVE D'ORNON Cedex, France.

Reporting and documentation

Guidelines on reporting and documentation are given in EPPO Standard PM 7/77 *Documentation and reporting on a diagnosis*.

Further information

Further information on this organism can be obtained from:

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Acknowledgements

This protocol was originally drafted for EPPO by: Dr Paola Del Serrone, Istituto Sperimentale per la Patologia Vegetale, Rome (IT).

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Appendix 1

Molecular methods

Sampling

The titer of phytoplasmas is usually low in woody hosts (Berges *et al*., 2000) and it varies in grapevine according to organ and season (Constable *et al*., 2003). Samples should be collected in July-September, selecting leaves showing symptoms but in good condition (no necrotic areas) and not affected by other pests. At least 20 leaves per plant should be randomly collected, and midribs and veins (to a total of about 1.5 g) separated. Material for diagnostic assays should be used fresh, or else ground in liquid nitrogen and stored at a maximum of –20°C (or lower depending on the storage time e.g. –80°C for more than two years).

DNA extraction

Several methods have been developed and compared (Palmano, 2001; Boudon-Padieu *et al*., 2003). Two methods are described below. The first one uses tissue disruption and phytoplasma enrichment (Ahrens & Seemüller, 1992) then DNA extraction is performed following a method from Doyle & Doyle (1990) and slightly modified by Marzachì *et al*., 1999. The second method which is more rapid consists of grinding grapevine tissues in CTAB buffer. Originally used at lower concentrations of Tris and CTAB for extraction of nucleic acids from periwinkle (Kollar *et al*., 1990), a CTAB concentration of 3% in 1M Tris (Boudon-Padieu *et al*., 2003) has been found to be optimum for grapevine and other woody plants that contain high quantities of phenolics, tannins and acids.

Phytoplasma enrichment procedure and DNA extraction

The following procedure has been validated during a ring test organised in Italy to establish a common protocol for DNA extraction from grapevine.

Grind 1.5 g of the fresh midveins in a sterile cold mortar and pestle with 7–8 mL of phytoplasma grinding buffer (PGB) freshly prepared (see Appendix 2) and 50 mg of sterile quartz sand (Sigma, cod. S9887). Incubate 10–15 min in ice. Add another 5 mL of PGB and homogenize thoroughly. Transfer into 15 mL tubes (Corex) and centrifuge in a pre-cooled rotor (Beckman JA 20) at 5000 rpm for 5 min (4°C). Transfer the supernatant in a cold clean Corex tube. Centrifuge at 19 000 rpm for 20 min (Beckman JA 20). Dry the pellet and re-suspend with 2 mL of 3% CTAB buffer (see Appendix 2), pre-warmed at 60°C. Incubate 10–20 min at 60°C with gentle agitation. Transfer 1 mL into a 2 mL clean Eppendorf tube and extract DNA by adding 1 mL of chloroform:isoamyl alcohol (24:1). Vortex and centrifuge at 6000 rpm for 10 min. Collect the aqueous phase and place it into a clean Eppendorf tube. Add 1 mL of cold isopropanol and incubate 5 min in ice. Centrifuge at 12 000 rpm for 20 min, discard the supernatant and wash the pellet with 1 mL of 70% ethanol. Centrifuge at 12 000 rpm for 10 min and dry the pellet. Re-suspend the pellet DNA in 400 µL TE (see Appendix 2) and precipitate in 900 µL of 95% ethanol and 40 µL of 3 M sodium acetate pH 5.2 at –80°C for 40 min or at –20°C overnight. Centrifuge at 12 000 rpm for 20 min, discard the supernatant and wash the pellet with 1 mL of 70% ethanol. Centrifuge at 12 000 rpm for 10 min and then re-suspend the pellet in 100 µL TE or dH_2O. As an alternative commercial kits (e.g. DNeasy, Qiagen) can be used for DNA extraction.

CTAB procedure for Nucleic acids extraction
(Boudon-Padieu *et al*., 2003)

This method was found to be almost as efficient as the above method in comparisons (Boudon-Padieu *et al*., 2003), however it is much more rapid and does not require multiple precipitation steps.

Nucleic acids can be extracted from fresh or frozen (–20°C or –80°C) tissues (preferably veins or petioles) of grapevine.

Grind 1 g of tissue using a ball-bearing apparatus in 7 mL of extraction buffer at room temperature. Transfer 1 mL of the suspension to an Eppendorf tube and incubate for 20 min at 65°C. Then add an equal volume of chloroform. Recover the aqueous phase and precipate the nucleic acids with an equal volume of cold isopropanol. Centrifuge to recover the precipitate, wash the pellet with 70% ethanol, dry and dissolve in 150 µL of TE buffer.

Amplification

a) Direct generic PCR followed by nested group-specific PCR

- Direct generic PCR

The P1/P7 primers are recommended (Deng & Hiruki, 1991; Schneider *et al*., 1995), with the following sequences:

P1: 5′-AAG AGT TTG ATC CTG GCT CAG GAT T-3′

P7: 5′-CGT CCT TCA TCG GCT CTT-3′

These primers amplify the whole length of 16S and intergenic 16S–23S and a small part of 23S rRNA gene.

Reaction mixture is as follows: 10X PCR buffer 5 µL; 25 mM $MgCl_2$ 3 µL, 10 mM dNTPs 4 µL; primer P1 20 µM 1 µL; primer P7 20 µM 1 µL; Taq polymerase 5 U $µL^{-1}$ 0.3 µL; DNA extract 2 µL diluted 1:10; water to 50 µL.

PCR is then conducted as follows: 1 cycle at 95°C for 3′; 35 cycles as follows: 94°C for 1 min, 50°C for 2 min; 72°C for 3 min; final extension 72°C for 5 min.

This step confirms the presence of a phytoplasma.

• Nested PCR with group 16SrV specific primers

This step uses the specific primers R16(V)F1 and R16(V)R1 (Lee *et al*., 1995), with the following sequences:

R16(V)F1: 5′-TTA AAA GAC CTT CTT CGG-3′

R16(V)R1: 5′-TTC AAT CCG TAC TGA GAC TAC C-3′

Reaction mixture and PCR cycles are as for the direct PCR, substituting the new primers in the same amounts. The DNA is provided as 2.0 µL of the product of the P1/P7 PCR, diluted 1:40. The product is visualized on a 1.0% agarose gel stained with ethidium bromide.

If necessary, RFLP analysis, using *Bfa I* enzyme, can be performed to distinguish grapevine flavescence dorée *sensu stricto* from other phytoplasmas belonging to the same group (elm yellow group), in particular Palatinate grapevine yellows.

b) Direct generic PCR followed by nested generic PCR and RFLP analysis

After the first generic PCR with primers P1 and P7 as described above, the obtained amplicon product is diluted 1:100 and used as the target in a nested PCR procedure with primers fU5 and rU3 (Lorenz *et al*., 1995) or with primers 16r758f (Gibb *et al*., 1995) and M23Sr (Padovan *et al*., 1995) with the following sequences:

fU5: 5′-CGG CAA TGG AGG AAA CT-3′

rU3: 5′-TTC AGC TAC TCT TTG TAA CA-3′

16f758f: 5′-GTC TTT ACT GAC GCT GAG GC-3′

M23Sr: 5′-TAG TGC CAA GGC ATC CAC TGT G-3′

PCR conditions are as follows:

Reaction mixture: 0.5 µM each primer, 0.25 mM each dNTP, 1.5 U Appligen *Taq* polymerase and buffer supplied with the enzyme, 1 µL of the diluted first PCR product, in a total reaction volume of 25 µL.

Amplification is carried out for 35 cycles under the conditions described in Table 2. PCR products are then submitted to digestion with restriction enzymes for phytoplasma group or subgroup identification.

RFLP analysis for characterization of phytoplasma group on the fU5/rU3 amplicon

Five to 20 µL of the fU5/rU3 amplification product is submitted to hydrolysis with *Tru9*I restriction enzyme at 65°C following the manufacturers instruction. Restriction products are analysed with 10% acrylamide gel electrophoresis and size of products is evaluated using MW ladder *pBR 322*/*Hae*III (Appligene) (Fig. 4).

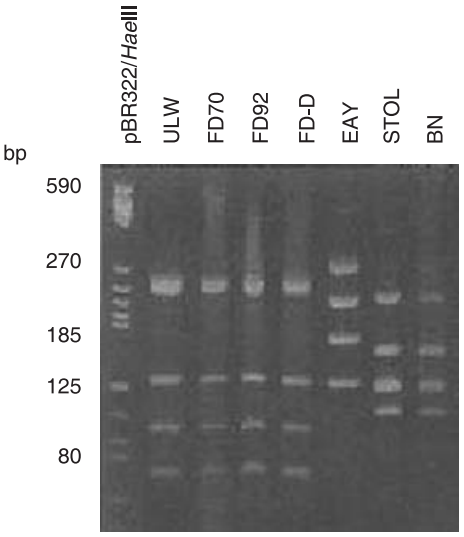

Fig. 4 Polyacrylamide gel (10%) RFLP analysis of phytoplasma rDNA fU5/rU3 fragment after digestion with *Tru*9I restriction enzyme. Lanes: pBR322/*Hae*III, Appligene ladder; ULW, elm yellows (Morvan isolate from France); FD70 and FD92, FD reference isolates from France; FD-D and FD-C, FD isolates from Italy (FD92 and FD-D are similar in all respects up to now); EAY, Eastern aster yellows; STOL, stolbur; BN, Bois noir –affected grapevine (stolbur phytoplasma).

RFLP analysis for characterization of 16SrV subgroup on the 16r758f/M23Sr amplicon

The 16r758f/M23Sr PCR product is submitted to a RFLP analysis with *Taq*I enzyme and the product is visualized with 10% acrylamide gel electrophoresis or 3% agarose gel, stained with ethidium bromide (Angelini *et al*., 2001) (Fig. 5). The size of the products is evaluated using MW ladder *pBR 322*/*Hae*III (Appligene).

c) Multiplex nested-PCR for simultaneous FD and BN detection

This bi-specific multiplex nested-PCR procedure was developed (Clair *et al*., 2003) to amplify simultaneously two non-ribosomal DNA fragments, of 1150 bp and 720 bp in length, which are specific for elm yellows-group (16SrV) and stolbur-group (16SrXII) phytoplasmas, respectively. Phytoplasma in groups 16SrV and 16SrXII are then identified

Table 2 Amplification conditions for direct generic PCR followed by nested generic PCR and RFLP analysis

Primer pair	Predenaturation	Denaturation	Annealing	Elongation	Final elongation
fU5/rU3	92°C 120 s	92°C 30 s	57°C 30 s	72°C 50 s	”
16r758f/M23Sr	92°C 120 s	92°C 60 s	50°C 120 s	72°C 180 s	”

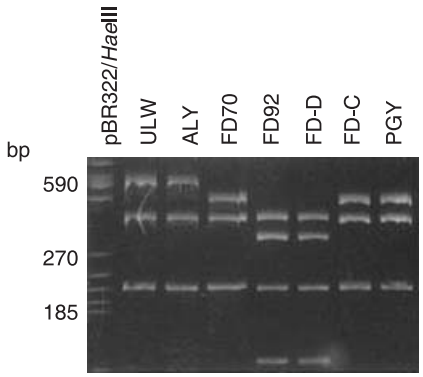

Fig. 5 Polyacrylamide gel (10%) RFLP analysis of rDNA fragment 16r758f/M23Sr after digestion with *Taq*I restriction enzyme. Lanes: pBR322/*Hae*III, Appligene ladder; ULW, elm yellows (Morvan isolate from France); ALY, alder yellows (Marcone isolate from Italy); FD70 and FD92, FD reference isolates from France; FD-D and FD-C, FD isolates from Italy (FD92 and FD-D are similar in all respects up to now); PGY, Palatinate grapevine yellows (Maixner PGY-A isolate), naturally transmitted from alder to grapevine by *Oncopsis alni* (Maxiner *et al*. 2000).

using agarose gel electrophoresis of amplification products on the basis of the size of the band obtained. The method is the official method in France (JORF N°112, May 14th, 2004, p. 08635). RFLP analysis of the FD9 product permits characterization of subgroup within group 16Sr V.

Sequences of primers for amplification of FD9 and STOL11 non-ribosomal DNA fragments are indicated in Table 3.

Reaction mixture for direct PCR are as follows: extracted DNA 1 µL, each dNTP 150 µM, primer FD9f1/r1 0.375 µM, primer STOL11f2/r1 0.0625 µM, Tris-HCl Buffer pH 9.0 10 mM, $MgCl_2$ 2.5 mM, KCl 50 mM, Triton X100 0.1%, BSA 0.2 mg mL^{-1}, Taq Polymerase (Q. Biogene) 0.2 U in a total volume of 20 µL.

Reaction mixture for Nested-PCR are as follows: extracted DNA,1 µL (1:1000 first amplification product), each dNTP 150 µM, Primer FD9f3b/r2 0.375 µM, primer STOL11f3/r2 0.375 µM, Tris-HCl Buffer pH 9.0 10 mM, $MgCl_2$ 2.5 mM, KCl 50 mM, Triton X100 0.1%, BSA 0.2 mg mL^{-1}, Taq Polymerase (Q. Biogene) 0.2 U in a total volume of 20 µL.

PCR conditions:

Predenaturation 92°C for 90 s, then 30 cycles for the first PCR or 35 cycles for the nested PCR, with denaturation 92°C for 40 s, hybridization 55°C for 40 s, elongation 72°C for 70 s. Separate amplicons with 1.2% Agarose gel electrophoresis, visualize with UV light after staining with ethidium bromide (Fig. 6).

RFLP analysis for characterization of the 16SrV subgroup on the FD9 amplicon

The FD9f3b/r2 PCR product is submitted to a RFLP analysis with *Tru*9I enzyme and the product is visualized after 10% acrylamide gel electrophoresis or 3% agarose gel, stained with ethidium bromide (Angelini *et al*., 2001) (Fig. 7).

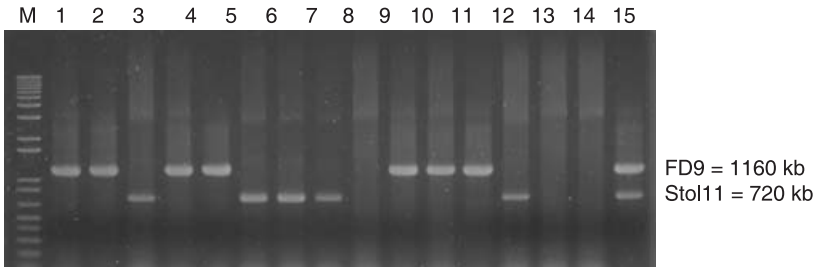

Fig. 6 Agarose gel electrophoresis of amplicons obtained with Multiplex-nested PCR for flavescence dorée and bois noir (BN) detection in grapevines. Lane M: 1 kb ladder. Lane 1–13: suspected GY-infected grapevines from different cultivars (FD: lane 1, 2, 4, 5, 10–12; BN: lane 3, 6, 7, 8, 13; double negative: lane 9). Lane 14: healthy grapevine extract. Lane 15: water control. Lane 16: double FD+stolbur (BN)-infected periwinkle (by D. Clair, INRA Dijon (FR), according to Clair *et al*., 2003).

Table 3 Sequences of primers for amplification of FD9 and STOL11 non-ribosomal DNA fragments

Primer name	Sequence	bp	Reference	Fragment size
FD9f1	5′-GAATTAGAACTGTTTGAAGACG-3′	22	Daire *et al*. (1997)	1300 bp
FD9r1	5′-TTTGCTTTCATATCTTGTATCG-3′	22	Daire *et al*. (1997)	
STOL11f2	5′-TATTTTCCTAAAATTGATTGGC-3′	22	Daire *et al*. (1997)	830 bp
STOL11r1	5′-TGTTTTTGCACCGTTAAAGC-3′	20	Daire *et al*. (1997)	
FD9f3b	5′-TAATAAGGTAGTTTTATATGACAAG-3′	25	Clair *et al*. (2003)	1150 bp
FD9r2	5′-GACTAGTCCCGCCAAAAG-3′	18	Angelini *et al*. (2001)	
STOL11f3	5′-ACGAGTTTTGATTATGTTCAC-3′	21	Clair *et al*. (2003)	720 bp
STOL11r2	5′-GATGAATGATAACTTCAACTG-3′	21	Clair *et al*. (2003)	

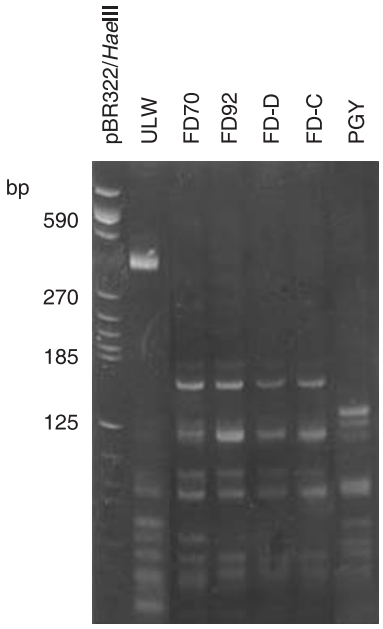

Fig. 7 Polyacrylamide gel (10%) RFLP analysis of 16SrV-group phytoplasma DNA fragment FD9 f3b/r2 after digestion with *Tru*9I restriction enzyme. Lanes : pBR322/*Hae*III, Appligene ladder; ULW, elm yellows (Morvan isolate from France); FD70 and FD92, FD reference isolates from France; FD-D and FD-C, FD isolates from Italy (FD92 and FD-D are similar in all respects up to now); PGY, Palatinate grapevine yellows (Maixner PGY-A isolate), naturally transmitted from alder to grapevine by *Oncopsis alni*.

Appendix 2

Buffers

Phytoplasma grinding buffer (PGB) for 100 mL
K_2HPO_4 anhydre, 1.67 g
KH_2PO_4, 0.41 g
Saccharose, 10 g
BSA (frac V), 0.15 g
PVP P.M. 10.000, 2 g
Ascorbic acid, 0.53 g
pH 7.6 with KOH drops.
Keep in ice until use.
CTAB buffer 3%
3% CTAB (cethyl-trimethyl-ammonium bromide) in 1 M Tris-HCl pH8
10 mM EDTA
1.4 M NaCl, 0.1% 2-mercaptoethanol.
TE buffer
10 mM Tris, 1 mM EDTA, pH 7.6.