

TECHNICAL MANUAL

Y Chromosome Deletion Detection System, Version 2.0

Instructions for Use of Product **MD1531**

For Research Use Only. Not for use in diagnostic procedures.



Y Chromosome Deletion Detection System, Version 2.0

All technical literature is available at: www.promega.com/protocols/
 Visit the web site to verify that you are using the most current version of this Technical Manual.
 E-mail Promega Technical Services if you have questions on use of this system: techserv@promega.com

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1. Description

The Y Chromosome Deletion Detection System, Version 2.0^(a,b), is intended for research use only and provides a rapid method for the detection of specific regions of the human Y chromosome. This system is designed to detect deletions occurring in YqAZF. Further mapping of common deletion breakpoints relative to palindromes 1 through 8 may be performed in additional experiments (1). This system consists of 20 primer pairs that are homologous to previously identified and mapped sequence-tagged sites (STS; 2–6). This product is not intended for use in diagnostic applications.

These primers will amplify nonpolymorphic short DNA segments from the Y chromosome when used in polymerase chain reactions (PCR; 1). The primers have been combined into five sets for use in multiplex PCR. This makes it possible to determine the presence or absence of all 20 sequence-tagged sites by performing five parallel PCR amplifications.

Four of the Multiplex Master Mix sets (Multiplexes A–D) contain a control primer pair that amplifies a fragment of the X-linked *SMCX* locus. The fifth Multiplex Master Mix set, Multiplex E, contains a control primer pair that amplifies a unique region in both male and female DNA (*ZFX/ZFY*). These control primer pairs are internal controls for the amplification reaction and the integrity of the genomic DNA sample. Finally, Multiplex E Master Mix includes a primer pair that amplifies a region of the *SRY* gene.

Figure 1 shows a typical amplification of male genomic DNA, as well as a negative DNA control, for each of the five Multiplex Master Mixes.

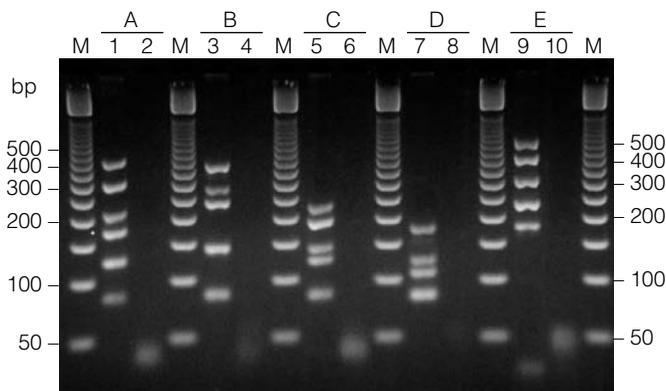


Figure 1. Multiplex analysis of wildtype male genomic DNA. Lane 1 shows the amplification products from reactions using Multiplex A Master Mix with male DNA, and lane 2 shows the no-DNA control. Lane 3 shows the amplification products from reactions using Multiplex B Master Mix with male DNA, and lane 4 shows the no-DNA control. Lane 5 shows the amplification products from reactions using Multiplex C Master Mix with male DNA, and lane 6 shows the no-DNA control. Lane 7 shows the amplification products from reactions using Multiplex D Master Mix with male DNA, and lane 8 shows the no-DNA control. Lane 9 shows the amplification products from reactions using Multiplex E Master Mix with male DNA, and lane 10 shows the no-DNA control. Lanes M contain the 50bp DNA Step Ladder (Cat. # G4521). The reactions were performed on a Perkin-Elmer Model 480 thermal cycler. The gel is a 4% NuSieve[®] 3:1 Plus TBE Buffer Reliant[®] gel.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
Y Chromosome Deletion Detection System, Version 2.0	25 reactions	MD1531

For Research Use Only. Not for use in diagnostic procedures. The Y Chromosome Deletion Detection System, Version 2.0, includes:

- 500µl Multiplex A Master Mix
- 500µl Multiplex B Master Mix
- 500µl Multiplex C Master Mix
- 500µl Multiplex D Master Mix
- 500µl Multiplex E Master Mix
- 200u GoTaq[®] DNA Polymerase
- 1ml Nuclease-Free Water
- 90µg 50bp DNA Step Ladder (340ng/µl)
- 2.5µg Male Genomic DNA (50ng/µl)
- 1ml Blue/Orange 6X Loading Dye

Storage Conditions: Store all components at –20°C. Avoid multiple freeze-thaw cycles. The Nuclease-Free Water may be stored at room temperature (22–25°C).

3. System Requirements

3.A. Template

DNA concentration, purity and size are important considerations to ensure success with the Y Chromosome Deletion Detection System, Version 2.0. DNA should be free of contaminating protein and salts. The DNA should not be sheared. Poor-quality DNA may result in increased background or amplification failure. Adding too much or too little DNA to the reaction can cause amplification failure. Failure can be manifested in several ways; sometimes complete lack of amplification of all bands is observed with all master mixes, and in other cases dropout of all or portions of the alleles in individual master mixes will be observed. Several commercially available DNA purification systems, including the Wizard[®] Genomic DNA Purification Kit (Cat.# A1120, A1125, A1620) and the MagneSil[®] KF, Genomic System (Cat.# MD1460), produce DNA of sufficient quality for the Y Chromosome Deletion Detection System.

Quantitation of DNA

We recommend quantitating the DNA prior to use in the Y Chromosome Deletion Detection System; either too much or too little DNA can cause the reactions to fail. Absorbance readings at 260nm can be used to estimate DNA concentration where 1Au = 50µg of double-stranded DNA/ml. Fifty nanograms of DNA should be used in each amplification reaction.

3.A. Template (continued)

DNA Quality

High-quality DNA typically has an A_{260}/A_{280} ratio of ≥ 1.8 . The presence of impurities in the DNA sample can cause amplification failure. We have observed that DNA concentration can be overestimated by spectrophotometry if the A_{260}/A_{280} ratio is low. When low amounts of DNA are added to the Y Chromosome Deletion Detection System, band dropout in one or more of the master mixes can be observed. The amount of DNA in a sample can be verified by ethidium bromide staining of DNA following electrophoresis in a 1% agarose gel. One hundred nanograms of genomic DNA should be visible on an ethidium bromide-stained gel visualized by UV transillumination. The Male Genomic DNA standard supplied in the Y Chromosome Deletion Detection System can be used as an electrophoresis standard. Figure 2 is an example of an agarose gel containing ethidium bromide-stained genomic DNA samples of 100ng of DNA as determined by A_{260} . Differences in the intensity of the bands indicate different DNA concentrations that result from variation in the A_{260}/A_{280} ratio.

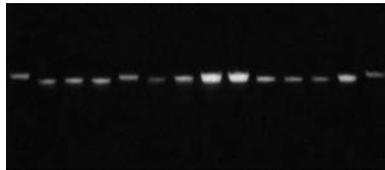


Figure 2. Gel image of 100ng (based on A_{260}) genomic DNA of varying quality. The end lanes contain 100ng of Male Genomic DNA supplied with the system.

3.B. Thermal Cyclers

The Y Chromosome Deletion Detection System has been optimized for use with either the Perkin-Elmer GeneAmp® PCR System 9600 or 9700 or the Perkin-Elmer Model 480 thermal cycler. Use of other thermal cyclers may require optimization of cycling parameters. We recommend validating with actual samples in addition to the positive control, as the preparation of the sample DNA can affect the cycling conditions. The Perkin-Elmer Model 480 thermal cycler program is a good starting point for thermal cyclers without programmed ramps, and the Perkin-Elmer GeneAmp® PCR System 9700 cycling program is a good starting point for thermal cyclers with programmed ramps.

3.C. Contamination Control

Preventing DNA contamination of reaction components is essential. Always use aerosol-resistant pipette tips and clean gloves, and avoid carryover contamination of stock solutions. Reagents that are used for amplification reactions should be maintained and used separately from completed amplification reactions, preferably in separate pre-amplification and post-amplification locations.

3.D. Control Reactions

Suitable control reactions should be included in each experiment.

- The positive Male Genomic DNA control should always yield the expected amplification products (see Section 7.E, Table 1, for expected sizes).
- The negative no-DNA control reaction will verify that the reagents are not contaminated with DNA (Figure 1; lanes 2, 4, 6, 8 and 10).

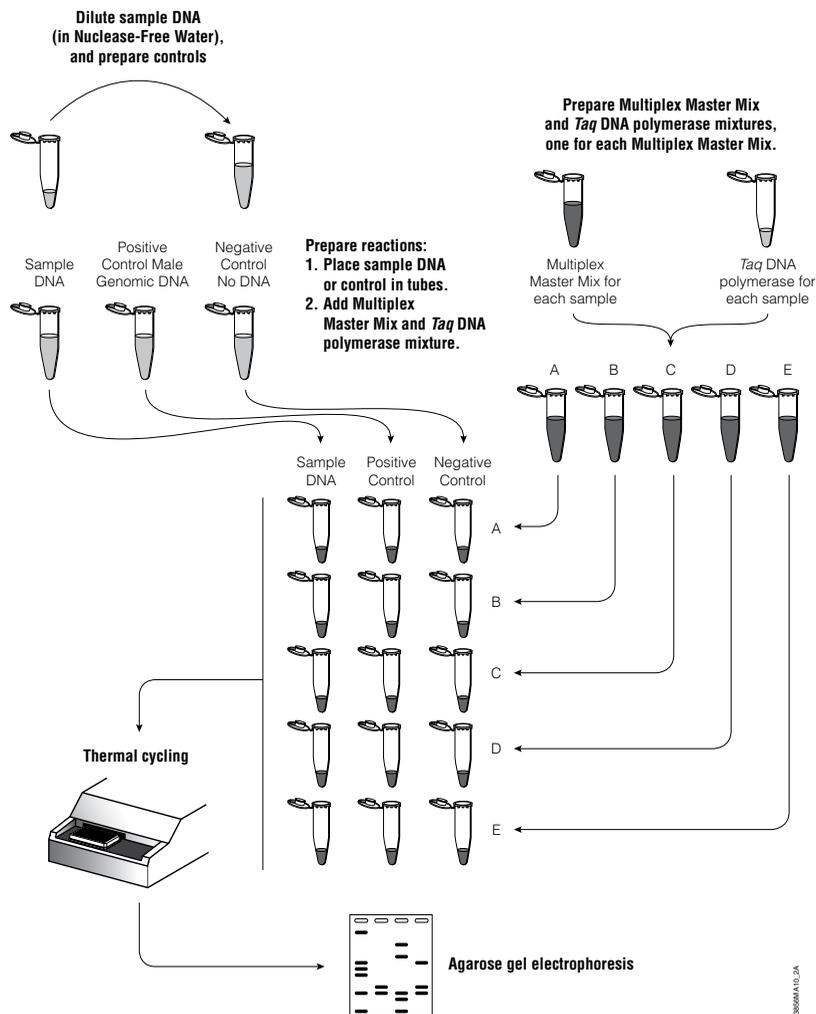


Figure 3. Schematic representation of the Y Chromosome Deletion Detection System assay.

4. Amplification Reactions

Materials to Be Supplied by the User

(Solution compositions are provided in Section 7.A.)

- nuclease-free light mineral oil
- Perkin-Elmer Model 480 or GeneAmp® PCR System 9600 or 9700 thermal cycler
- Amplification tubes compatible with thermal cycler. For the Perkin Elmer Model 480 thermal cycler, use 0.5ml thin-walled GeneAmp® reaction tubes. For the GeneAmp® PCR System 9600 and 9700 thermal cyclers, use 0.2ml thin-walled MicroAmp® reaction tubes or a MicroAmp® optical 96-well reaction plate.
- 4% NuSieve® 3:1 Plus TBE Buffer agarose precast Reliant® minigels (Cambrex
- Cat.# 54927, 54928 or 54929), Latitude® precast minigels agarose (Cambrex
- Cat.# 57222 or 57232), or NuSieve® 3:1 agarose (Cambrex Cat.# 50090 or 50091)
- 1X TBE buffer
- ethidium bromide
- aerosol-resistant pipette tips
- UV transilluminator



Caution: Ethidium bromide is a suspected carcinogen. Always wear gloves when working with ethidium bromide solutions.

4.A. Reaction Setup for Large Sample Numbers

This protocol is designed to analyze multiple samples at one time. If you are analyzing only 1 or 2 samples, see Section 7.C.

In this procedure:

- Aliquots of the sample DNAs are placed in the reaction tube.
- *Taq* DNA polymerase is added to each of the Multiplex Master Mixes.
- The Multiplex Master Mixes containing *Taq* DNA polymerase are added to the reaction tubes. Each sample is analyzed with all five Multiplex Master Mixes.

Protocol

1. Thaw the Multiplex Master Mixes. Vortex for 5–10 seconds, and store on ice. Thaw the Nuclease-Free Water and Male Genomic DNA.
2. Determine the number of reactions for each Multiplex Master Mix. For each sample there will be five reaction tubes, one for each Multiplex Master Mix. Include a positive Male Genomic DNA control and a negative no-DNA control for each Multiplex Master Mix.

Multiplex Master Mix	# of Sample DNAs	Positive Control (Male Genomic DNA)	Negative Control (No DNA)	Total Reaction Tubes
A		1	1	
B		1	1	
C		1	1	
D		1	1	
E		1	1	

- Set up and label the required number of reaction tubes as determined above. Use thin-walled amplification tubes or an optical plate. Place on ice.
- In a separate tube, dilute each sample DNA to 10ng/μl using the supplied Nuclease-Free Water. Mix well by vortexing for 5–10 seconds. Add 5μl of diluted DNA to the appropriately labeled reaction tubes (above) on ice. Dilute the Male Genomic DNA to 10ng/μl by adding 6μl of Male Genomic DNA to 24μl of Nuclease-Free Water. Mix well by vortexing for 5–10 seconds. Add 5μl to appropriately labeled reaction tubes (above) on ice. For the negative control (no DNA), add 5μl of Nuclease-Free Water to the appropriately labeled reaction tubes (above) on ice.
- Prepare five Multiplex Master Mix and *Taq* DNA polymerase mixtures on ice, one for each Multiplex Master Mix. Vortex the Multiplex Master Mixes before using them.

Component	Volume per Reaction	Volume per 10 Reactions
Multiplex Master Mix	20μl	200μl
<i>Taq</i> DNA Polymerase (5u/μl)	0.2μl	2μl
Final Volume	20.2μl	202μl

- Vortex to mix.
- Add 20μl of the Multiplex Master Mix and *Taq* DNA polymerase mixture to the appropriate reaction tubes, which contain the sample DNA or controls, on ice.
- Gently vortex to mix.
- Centrifuge briefly to bring the contents to the bottom of the tubes. Place on ice until ready for thermal cycling.
- If using a thermal cycler that requires mineral oil, tilt the tubes and add one drop of oil to the side of the tube, letting the oil run down the side of the tube.



4.B. Thermal Cycling: Protocols for use with the Perkin-Elmer Model 480 and GeneAmp® PCR System 9600 or 9700 Thermal Cyclers

Place the tubes in the thermal cycler, and run the recommended program. The preferred protocols for use with the Perkin-Elmer Model 480 and GeneAmp® PCR System 9600 and 9700 thermal cyclers are provided below. It may be necessary to optimize the program with other thermal cyclers.

! **Note:** It is important to preheat the instrument to 94°C before placing tubes into the machine.

Program for the Perkin-Elmer Model 480 Thermal Cycler

Preheat the thermal cycler to 94°C before placing tubes inside.

Cycling Profile:

94°C for 2 minutes, then:

94° for 1 minute

57°C for 30 seconds

72°C for 1 minute

For **35 cycles**, then:

72°C for 5 minutes

4°C soak

After completion of the thermal cycling protocol, store the samples at –20°C.

Program for the Perkin-Elmer GeneAmp® PCR System 9600 Thermal Cycler

Preheat the thermal cycler to 94°C before placing tubes inside.

Cycling Profile:

94°C for 2 minutes, then:

94°C for 30 seconds

Ramp 68 seconds to 58°C, hold 30 seconds

Ramp 50 seconds to 70°C, hold 45 seconds

For **35 cycles**, then:

68°C for 2 minutes

4°C soak

After completion of thermal cycling protocol, store samples at –20°C.

Program for the Perkin-Elmer GeneAmp® PCR System 9700 Thermal Cycler

Preheat the thermal cycler to 94°C before placing tubes inside.

Cycling Profile:

- 94°C for 2 minutes, then,
- 94°C for 30 seconds
- 30% Ramp to 58°C, hold 30 seconds
- 30% Ramp to 70°C, hold 45 seconds
- Repeat for **35 cycles**, then:
- 68°C for 2 minutes
- 4°C soak
- (9600 default mode)

After completion of the thermal cycling protocol, store samples at –20°C.

4.C. Agarose Gel Electrophoresis

1. For optimal visualization of the amplification products we recommend using a 4% NuSieve® 3:1 Plus TBE buffer precast gel. Alternatively, cast a 4% NuSieve® 3:1 agarose gel in 1X TBE buffer containing 0.5µg/ml ethidium bromide. For instructions for the preparation of agarose gels, see Section 7.D.



Note: The running buffer should contain 0.5µg/ml ethidium bromide.

2. Dilute the molecular weight marker as follows:

	Volume
50bp DNA Step Ladder	12µl
Blue/Orange 6X Loading Dye	4µl
Nuclease-Free Water	8µl

3. Add 2.5µl of the Blue/Orange 6X Loading Dye to each amplification tube, and mix.
4. Load 5–10µl of each sample and 10µl of the diluted molecular weight markers onto the gel.
5. Run the gel in 1X TBE buffer containing 0.5µg/ml ethidium bromide at 5V/cm (measured as the distance between the electrodes) until the bromophenol blue dye front migrates to the bottom of the gel.
6. Photograph the gel using a UV transilluminator (320nm).

4.D. Data Analysis—Controls

Determine that the control reactions produced the expected results before analyzing your samples. The worksheets provided (see Section 7.E, Table 1) can be used for analysis of both the control and experimental samples.

1. **Negative No-DNA Control:** There should be no specific amplification products in the lanes containing the negative no-DNA control reactions. There may be some low-molecular-weight bands or smearing that are the result of primer interactions. Amplification products in the negative no-DNA control reactions are indicative of contaminating DNA, and the experiment should be repeated with care to avoid contamination.
2. **Positive Male Genomic DNA Control:** The number and size of the amplification products for each Multiplex Master Mix are indicated in Table 1 (Section 7.E). The positive Male Genomic DNA control reaction for each Multiplex Master Mix should have all the bands indicated for that Multiplex Master Mix (Table 1). The sizes of the amplification products can be estimated by comparison with the DNA markers. If all of the expected bands are not present in the reactions with positive Male Genomic DNA control, or if there are prominent extra bands, it is indicative of a problem with the amplification reagents or the thermal cycler (see Section 5). Results should not be considered valid if any of the expected amplification products are missing in the reactions with the positive Male Genomic DNA control.
3. **Control Primer in Multiplex Master Mixes:** In Multiplexes A, B, C, and D reactions the smallest amplification product (83bp) should be produced from an X-linked locus (*SMCX*). In Multiplex E the largest (496bp) amplification product should be produced from the *ZFY/ZFX* genes. The absence of these products is indicative of a problem with that particular PCR amplification. If the control bands are present with the Male Genomic DNA control but not with the sample DNA, it suggests that there may be a problem with the genomic DNA used as a template, such as the presence of impurities, inaccurate DNA quantitation or degraded DNA. Check the DNA template on an agarose gel before repeating the amplification. Repeat any reactions in which the control product is absent. It may be necessary to isolate the template DNA again. We recommend using the Wizard® Genomic DNA Purification Kit (Section 7.B; Cat.# A1120, A1125, A1620) or the MagneSil® KF, Genomic System (Cat.# MD1460) for isolation of template DNA. Ideally, DNA should be eluted in water.
4. **Female DNA Control (optional):** Some researchers include a female DNA control. For Multiplexes A, B, C and D, the smallest product (83bp) and the largest product (496bp) for Multiplex E should be present when female DNA is used as a template. Nonspecific bands may appear when female genomic DNA is used. These bands are generally faint and do not correspond in size to male-specific bands. With Multiplex E, a distinct high-molecular-weight band is generally seen.

4.E. Data Analysis—Experimental Samples

The worksheets provided in the Appendix, Section 7.E, can be used for analysis of experimental samples. Determine the presence or absence of the expected PCR products (Table 1). If there are any products absent from the reactions, they can be mapped using the Y Chromosome Map Worksheet (Table 2). All deletions should be contiguous. Figure 4 shows an example of gel analysis of DNA carrying a Y chromosome deletion.

Adjacent regions of the Y chromosome do not appear as sequential amplification products using the Y Chromosome Deletion Detection System. Exceptions are SY242 and SY208 of the DAZ locus, which are represented as sequential amplification products in Multiplex B, and SY84 and SY86 of the DYS273 and DYS148 loci, which are represented as sequential amplification products in Multiplex E.

If multiple amplification bands are missing, and those bands do not map to adjacent regions of the Y chromosome (Table 2), they represent dropout bands and are not deletions. See the troubleshooting guide (Section 5) for help if you observe dropout of alleles in individual master mixes.

We have observed some nonspecific bands that appear above or below the expected amplification products on the agarose gel. If your negative controls show no detectable PCR products, these nonspecific bands should have no effect on analysis.

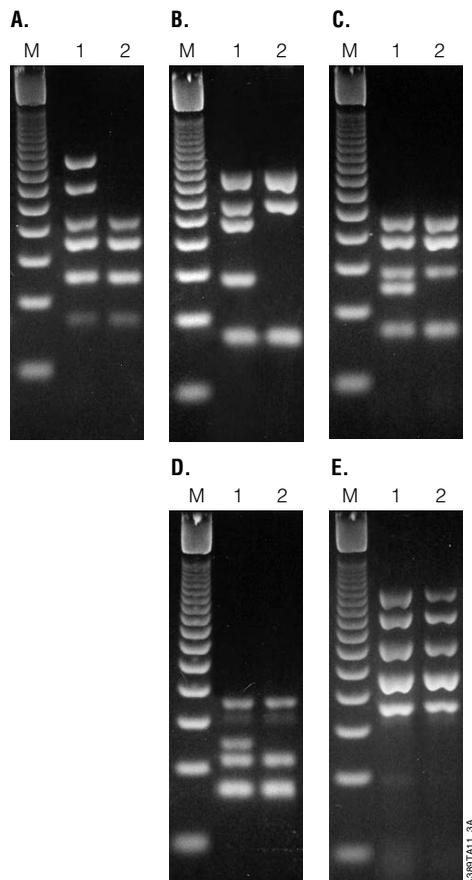


Figure 4. Y chromosome deletion analysis. The amplification products from Multiplex A Master Mix, Multiplex B Master Mix, Multiplex C Master Mix, Multiplex D Master Mix and Multiplex E Master Mix reactions are shown. Lanes: 1, Male Genomic DNA control; 2, sample male DNA (containing deletions). Bands generated with Male Genomic DNA control are compared to those generated with a sample containing Y chromosome deletions. (Note the deleted bands in Multiplex A–D. Multiplex E shows no deletions.) The marker (M) lanes contain the 50bp DNA Step Ladder (Cat.# G4521).

5. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: www.promega.com; E-mail: techserv@promega.com

Symptoms

Low yield or no amplification product

Causes and Comments

Thermal cycler programmed incorrectly. Verify that the times and temperatures are correct.

Thermal cycler not properly calibrated. Perform a set of control reactions to determine if certain positions in the thermal cycler yield little or no product.

Top of thermal cycler open. The top must be closed for correct heating and cooling.

Missing reaction component. Check the reaction components, and repeat the reaction.

Degraded reagent. Store Multiplex Master Mixes at -20°C . Keep on ice once thawed. Avoid multiple freeze-thaw cycles.

Incorrect tubes. Use only thin-walled microcentrifuge tubes for amplification. For the Perkin-Elmer Model 480 thermal cycler, use 0.5ml thin-walled GeneAmp[®] reaction tubes, and for the GeneAmp[®] PCR System 9600 or 9700 thermal cycler, use 0.2ml thin-walled MicroAmp[®] reaction tubes or MicroAmp[®] optical 96-well reaction plate.

Inhibitors in the DNA sample. DNA should be free of contaminating proteins and salts. Repurify or ethanol precipitate the DNA sample to remove inhibitors.

Too much or too little DNA used. DNA should be quantitated prior to use in the Y Chromosome Deletion Detection System. Use 50ng of high-molecular-weight (nonsheared) DNA for each amplification reaction or 250ng total for each sample.

Inaccurate quantitation of DNA. DNA concentration can be overestimated by spectrophotometry if the A_{260}/A_{280} ratio is low. Verify DNA concentration and quality by ethidium bromide staining after electrophoresis on a low percent (1–1.2%) agarose gel. We recommend comparing sample DNA with genomic DNA of known concentration such as the Male Genomic DNA supplied with the system. One hundred nanograms of the Male Genomic DNA is clearly visible on ethidium bromide-stained gels.

Poor mixing of components. Concentration gradients can form in Multiplex Master Mixes stored at -20°C . Mix thoroughly by vortexing for 10–15 seconds before use.

Symptoms	Causes and Comments
Low yield or no amplification product (continued)	The thermal cycler was not preheated prior to placing tubes inside. Preheat the instrument to 94°C.
Multiple nonspecific amplification products	Thermal cycler programmed incorrectly. Verify that the times and temperatures are correct.
	Reactions were not set up on ice. Make sure to set up reactions on ice.
	Too much genomic DNA. We recommend using 50ng of DNA per reaction.
	Too much <i>Taq</i> DNA polymerase. Do not use more than 1 unit of <i>Taq</i> DNA polymerase per reaction.
	Annealing temperature too low. Check the accuracy of the thermal cycler. Increase the annealing temperature in 0.5–1°C increments.
Missing bands in control reaction	Annealing temperature too high. Check the accuracy of the thermal cycler. Decrease the annealing temperature in 0.5–1°C increments.
Internal control band is missing in sample DNA but present in control	Sample DNA degraded or of poor quality. Repeat reactions. You may need to re-isolate sample DNA. We recommend the Wizard® Genomic DNA Purification Kit or MagneSil® KF, Genomic System.
Dropout of bands in amplification of sample DNA	<p>Too little DNA used. DNA should be quantitated prior to use in the Y Chromosome Deletion Detection System. Use 50ng for each amplification reaction or 250ng total for each sample. DNA concentration can be overestimated by spectrophotometry if the A_{260}/A_{280} ratio is low. Verify DNA concentration by ethidium bromide staining after electrophoresis on a low percent (1–1.2%) agarose gel. We recommend comparing sample DNA with genomic DNA of known concentration such as the Male Genomic DNA supplied with the system. One hundred nanograms of the Male Genomic DNA is clearly visible on ethidium bromide-stained gels.</p> <p>Poor DNA purity. High-quality DNA typically has an A_{260}/A_{280} ratio of ≥ 1.8. However, A_{260}/A_{280} ratio may not accurately predict purity. Impurities in the sample may cause amplification failure. To test for impurities, add some of your sample DNA to the positive control. Subsequent inhibition of this “spiked” positive control reaction indicates that impurities are in the sample DNA.</p>

5. Troubleshooting (continued)

Symptoms	Causes and Comments
Dropout of bands in amplification of sample DNA (continued)	<p>Suboptimal conditions. The system is tested using the Perkin-Elmer GeneAmp® PCR System 9600, 9700 and the Model 480 thermal cyclers. If you are using a different thermal cyclers, you will need to optimize amplification conditions.</p> <p>EDTA in sample. EDTA at a concentration of $\geq 0.4\text{mM}$ can adversely affect amplification.</p> <p>Incorrect protocol. The protocol must be followed precisely.</p> <p>Wrong reaction volume. Standard reactions are 25μl. Reactions of 12.5μl are prone to dropout of multiple bands.</p>
Bands in negative control reactions	<p>Cross-contamination. Pre- and post-amplification areas should be isolated, preferably in separate rooms. Use aerosol-resistant tips and dedicated pipettes for assembling reactions. Workstations and pipettes can be cleaned with a mild bleach solution before and after use.</p>

6. References

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* Especially note Figure 2, available online as data supplementary to the article.
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10. Repping, S. *et al.* (2002) Recombination between palindromes P5 and P1 on the human Y chromosome causes massive deletions and spermatogenic failure. *Am. J. Hum. Genet.* **71**, 906–22.
11. Saxena, R. *et al.* (1996) The DAZ gene cluster on the human Y chromosome arose from an autosomal gene that was transposed, repeatedly amplified and pruned. *Nat. Genet.* **14**, 292–9.

7. Appendix

7.A. Composition of Buffers and Solutions

1X TBE buffer

89mM	Tris-base
110mM	boric acid
2mM	EDTA

7.B. Related Products

Accessory Components

Product	Size	Cat.#
Nuclease-Free Water	(2 × 25ml) 50ml	P1193
Mineral Oil	12ml	DY1151

DNA Purification Systems

Product	Size	Cat.#
Wizard® Genomic DNA Purification Kit	100 isolations × 300µl	A1120
	500 isolations × 300µl	A1125
	100 isolations × 10ml	A1620
MagneSil® KF, Genomic System	200 preps	MD1460

7.C. Reaction Setup for Small Sample Numbers

This protocol is designed to analyze a small number of samples. The order of addition of *Taq* DNA polymerase, Multiplex Master Mixes and genomic DNA is different from the Large Sample Number Protocol (Section 4.A). These changes conserve the Multiplex Master Mixes to ensure the full number of reactions can be performed.

In this procedure:

- The Multiplex Master Mixes are added to the reaction tubes.
- A mixture of genomic DNA and *Taq* DNA polymerase in Nuclease-Free Water is prepared.
- The genomic DNA/*Taq* DNA polymerase mixture is added to each Multiplex Master Mix.
- Each sample DNA is analyzed with all five of the Multiplex Master Mixes.

7.C. Reaction Setup for Small Sample Numbers (continued)

An alternative protocol for large sample numbers is also provided (Section 4.A).

1. Thaw the Multiplex Master Mixes. Vortex for 5–10 seconds, and store on ice. Thaw the Nuclease-Free Water and Male Genomic DNA.
2. Determine the number of reactions to perform for each Multiplex Master Mix. For each sample there will be five reaction tubes, one for each Multiplex Master Mix. Include a positive Male Genomic DNA control and negative no-DNA control for each Multiplex Master Mix.

Multiplex Master Mix	# of Sample DNAs	Positive Control (Male Genomic DNA)	Negative Control (No DNA)	Total Reaction Tubes
A		1	1	
B		1	1	
C		1	1	
D		1	1	
E		1	1	

3. Label the required number of reaction tubes as determined above. For the Perkin-Elmer Model 480 thermal cycler, use 0.5ml thin-walled GeneAmp® reaction tubes, and for the GeneAmp® PCR System 9600 or 9700 thermal cycler, use 0.2ml thin-walled MicroAmp® reaction tubes or MicroAmp® optical 96-well reaction plate. Place the reaction tubes on ice.
4. Vortex the Multiplex Master Mixes for 5–10 seconds. Place 20µl of each Multiplex Master Mix in the appropriately labeled reaction tubes on ice.
5. For each DNA sample, prepare a genomic DNA mixture on ice following the table below.

Component	Single Reactions	Duplicate Reactions
Sample DNA	~250ng	~500ng
<i>Taq</i> DNA polymerase (5u/µl)	1µl	2µl
Nuclease-Free Water to a final volume of	25µl	50µl

For the positive Male Genomic DNA control reactions, combine 5µl of Male Genomic DNA, 1µl of *Taq* DNA polymerase (5u/µl) and 19µl of Nuclease-Free Water on ice. Vortex to mix.

For the negative no-DNA control, add 1µl *Taq* DNA polymerase (5u/µl) to 25µl of Nuclease-Free Water on ice. Vortex gently to mix.

6. Add 5µl genomic DNA or control mixture prepared in Step 5 to the appropriate reaction tubes containing the Multiplex Master Mixes on ice. Vortex to mix.

7. Centrifuge briefly to bring the contents to the bottom of the tubes. Place reaction tubes on ice until ready for thermal cycling.
8. If using a thermal cycler that requires mineral oil, tilt the tubes and add one drop of oil to the side of the tube, letting the oil run down the side of the tube.

7.D. Preparation of Agarose Gels

1. Use NuSieve® 3:1 agarose available from Cambrex.
2. Choose a beaker 2–4 times the volume of the agarose solution.
3. Add the appropriate amount of NuSieve® 3:1 agarose and 1X TBE buffer to make a 4% gel (i.e., 100ml of 1X TBE buffer and 4g NuSieve® 3:1 agarose) to the beaker.
4. Weigh the beaker, or mark the level of the liquid in the beaker.
5. Mix to wet agarose.
6. Soak the agarose for 15 minutes at room temperature.
7. Heat the beaker in a microwave until the solution boils.
8. Boil the agarose for 1 minute or until it is completely dissolved.
9. Remove the agarose from the microwave.
10. Gently swirl the beaker to mix.



Caution: Any microwaved solution may become superheated and boil over when agitated.

11. Add sufficient hot distilled water to obtain the initial weight or volume.
12. Mix thoroughly.
13. Cool the solution to 50–60°C.
14. Add ethidium bromide to 0.5µg/ml.
15. Pour the gel to a depth of 5mm. It is important that the gel is thin for optimum resolution.
16. Allow the gel to set.
17. Run gel in 1X TBE buffer containing 0.5µg/ml ethidium bromide. For best resolution, it is important ethidium bromide is in both the running buffer and the gel.
18. Load samples.
19. Run gel at 5V/cm until the bromophenol blue dye front migrates to the bottom of the gel.



7.E. Worksheets

Table 1. PCR Amplification Product Profile for Test Samples.

Record the presence (+) or absence (-) of the PCR amplification products for each experimental sample.

Multiplex A Master Mix				Samples (+/-)					
STS	Locus	Size of Product (bp)	Map Position						
SY254	<i>DAZ</i>	380	18						
SY157	DYS240	290	20						
SY81	DYS271	209	2						
SY130	DYS221	173	11						
SY182	<i>KAL-Y</i>	125	5						
	<i>SMCX</i>	83	Control						

Multiplex B Master Mix				Samples (+/-)					
STS	Locus	Size of Product (bp)	Map Position						
SYPR3	<i>SMCY</i>	362	7						
SY127	DYS218	274	9						
SY242	<i>DAZ</i>	233	16						
SY208	<i>DAZ</i>	140	17						
	<i>SMCX</i>	83	Control						

Multiplex C Master Mix				Samples (+/-)					
STS	Locus	Size of Product (bp)	Map Position						
SY128	DYS219	228	10						
SY121	DYS212	190	6						
SY145	DYF51S1	143	14						
SY255	<i>DAZ</i>	124	19						
	<i>SMCX</i>	83	Control						

Multiplex D Master Mix				Samples (+/-)					
STS	Locus	Size of Product (bp)	Map Position						
SY133	DYS223	177	12						
SY152	DYS236	125	15						
SY124	DYS215	109	8						
	<i>SMCX</i>	83	Control						

Multiplex E Master Mix				Samples (+/-)					
STS	Locus	Size of Product (bp)	Map Position						
	<i>ZFX/ZFY</i>	496	Control						
SY14	<i>SRY</i>	400	1						
SY134	DYS224	303	13						
SY86	DYS148	232	3						
SY84	DYS273	177	4						

8. Summary of Changes

The following change was made to the 12/18 version of this technical manual:

The document design was updated.

^{a)}U.S.Pat. No. 6,242,235, Australian Pat. No. 761757, Canadian Pat. No. 2,335,153, Chinese Pat. No. ZL99808861.7, Hong Kong Pat. No. HK 1040262, Japanese Pat. No. 3673175, European Pat. No. 1088060 and other patents pending.

^{b)}Use of this product for basic PCR is outside of any valid US or European patents assigned to Hoffman La-Roche or Applera. This product can be used for basic PCR in research, commercial or diagnostic applications without any license or royalty fees.

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