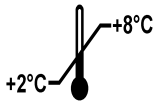
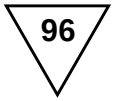
ProntoPlex™ AAT kit

For the detection of the PiS and PiZ alleles of the alpha-1-antitrypsin gene

Instructions for Use

REF 9965





PRONTO DIAGNOSTICS

IVD CE

INTENDED USE

The ProntoPlex™ AAT kit is a single nucleotide primer extension ELISA assay intended for the qualitative *in vitro* detection of the PiS and PiZ alleles in the alpha-1-antitrypsin gene, from amplified human DNA.
For *in vitro* diagnostic use.

BACKGROUND

Alpha1-antitrypsin (AAT) deficiency is the most prevalent potentially lethal hereditary disease of Caucasians. It leads to jaundice in infants, liver disease in children and adults, and pulmonary emphysema in adults.
AAT is a protease inhibitor which protects tissue structures from damage by degrading enzymes. The genetic defect in AAT deficiency results in a molecule that cannot be released from its production site in hepatocytes. Low serum levels of AAT result in low alveolar concentrations, where the protein normally would serve as protection against proteases. Consequential protease excess destroys alveolar walls and causes obstructive lung disease. Moreover, unsecreted AAT self-aggregates in the liver and causes liver disease.
Mutations in the PI (protease inhibitor) locus, located on chromosome 14, are associated with AAT deficiency. The most common risk alleles are PiS, whose worldwide carrier rate is 1:50 (1:9 to 1:12 in Caucasians) and PiZ, with a worldwide carrier rate of 1:162 (1:30 to 1:40 in Caucasians).

REFERENCES

1. Eriksson, S. Pulmonary emphysema and alpha1-antitrypsin deficiency. *Acta Med. Scand*., 175:197-205 (1964).
2. Laurell, C.B. and Eriksson, S. The electrophoretic alpha-1-globulin pattern of serum in alpha-1-antitrypsin deficiency. *Scand. J. Clin. Lab. Invest.,* 15:132-140 (1963).
3. De Serres. F.J. Worldwide Racial and Ethnic Distribution of alpha 1 Antitrypsin Deficiency. *Chest,* 122:1818-1829 (2002).
4. Niewoehner, D.E. Cigarette smoking, lung inflammation, and the development of emphysema. *J. Lab. Clin. Med.,* 111 :15-27 (1988).
5. Brantly, M., Nukiwa, T., and Crystal, R.G. Molecular basis of alpha-1-antitrypsin deficiency. *Am. J. Med.,* 84 (suppl. 6A):13-31 (1988).
6. Crystal, R.G., Brantly, M.L., Hubbard, R.C., Curiel, D.T., States, D.J., and Holmes, M.D. The alpha 1-antitrypsin gene and its mutations: Clinical consequences and strategies for therapy. *Chest*, 95:196-206 (1989).

WARNINGS & PRECAUTIONS

- Reagents supplied in this kit may contain up to 0.1% sodium azide that is toxic if swallowed. Sodium azide has been reported to form explosive lead or copper azides in laboratory plumbing. To prevent the accumulation of these compounds, flush the sink and plumbing with large quantities of water.
- TMB Substrate solution is an irritant of the skin and mucous membranes. Avoid direct contact.
- The Stop Solution contains dilute sulfuric acid (1M), which is an irritant of the eyes and the skin. In case of contact with the eyes, immediately flush them with water. Do not add water to this product. In case of an accident or discomfort consult a physician (if possible, show the bottle label).
- In addition to reagents in this kit, the user may come in contact with other harmful chemicals that are not provided, such as ethidium bromide and EDTA. The appropriate manufacturers' Material Safety Data Sheets (MSDS) should be consulted prior to the use of these compounds.

ASSAY OVERVIEW

The PRONTO® procedure detects predefined polymorphisms in DNA sequences, using a single nucleotide primer extension ELISA assay.
The ProntoPlex™ AAT kit uses a multiplex primer extension reaction, which detects both the PiS and PiZ alleles in a single microplate well.
A complete genotype of positive samples can be confirmed using the PRONTO® AAT Verification Strip.

TARGET DNA AMPLIFICATION: The DNA fragments that encompass the tested mutations are amplified. This amplified DNA is the substrate for the primer extension reaction.

2 **POST-AMPLIFICATION TREATMENT:** The amplified DNA is treated to inactivate free unincorporated nucleotides, so that they will not interfere with the primer extension reaction.

3 **PRIMER EXTENSION REACTION:** A single-nucleotide primer extension reaction is carried out in a 96-well thermoplate. Each well contains a 5'-labeled primer that hybridizes to the tested DNA next to the suspected mutation site, and a single biotinylated nucleotide species, which complements the nucleotide base at the tested site. Each post-amplification treated sample is tested in two wells per mutation: the first well of each pair tests for the presence of the mutant allele (*mut*), while the second well tests for the presence of the normal allele (*wt*). The biotinylated nucleotide will be incorporated to the primer in the course of the reaction or not added, depending on the tested individual's genotype.

4 **DETECTION BY ELISA:** The detection of the biotinylated primers is carried out by an ELISA procedure: The biotin-labeled primers bind to a streptavidin-coated ELISA plate and are detected by a peroxidase-labeled antibody (HRP) directed to the 5' antigenic moiety of the primer. A peroxidase reaction occurs in the presence of TMB-Substrate.

5 **INTERPRETATION OF THE RESULTS:** The results are determined either visually (substrate remains clear or turns blue) or colorimetrically (substrate remains clear or turns yellow) following the addition of the stop solution.

DISCLAIMER

- Results obtained using this kit should be confirmed by an alternative method.
- Confirmed results should be used and interpreted only in the context of the overall clinical picture. The manufacturer is not responsible for any clinical decisions that are taken.

The user of this kit should emphasize these points when reporting results to the diagnosing clinician or the genetic counselor.

CONTENTS OF THE KIT

Amplification Mix AAT	1 vial	(1,600 µL)
PRONTO® Buffer 2	1 bottle	(3 mL)
Solution C	1 vial (yellow cap)	(130 µL)
Solution D	1 vial (red cap)	(100 µL)
ColoRed™ Oil	1 dropper bottle	(13 mL)
Assay Solution	1 bottle (green solution)	(100 mL)
Wash Solution (conc. 20x)	1 bottle	(100 mL)
HRP Conjugate	1 vial	(450 µL)
TMB Substrate	1 bottle	(40 mL)
Stop Solution (1M H_2SO_4)	1 bottle	(30 mL)
ProntoPlex™ AAT Plates	2 individually pouched plates	
Detection Plates	2 Strepatividin-coated ELISA plates	
PRONTO® AAT Verification Strips	2 individually pouched strips	

STORAGE AND STABILITY

- Store at 2-8ºC. **Do not freeze**.
- Do not use the kit beyond its expiration date (marked on box label). Stability is maintained even when components are re-opened several times.
- Minimize the time reagents spend at room temperature.
- This kit has been calibrated and tested as a unit; **do not mix reagents from kits with different lot numbers.**

ADDITIONAL MATERIALS REQUIRED

- Deionized water (about two liters per kit)
- Thermowell plate or tubes (thin wall) for the post-amplification treatment
- Sterile pipette tips
- Troughs/reagent reservoirs - for use with the detection reagents
- Thermocycler for a 96-well microplate
- Multichannel pipettes (5-50 µL and 50-200 µL)
- Positive displacement pipettes (1-5 µL, 5-50 µL, 50-200 µL & 200-1,000 µL)
- Filtered tips
- ELISA reader with 450 nm filter (optional - 620 nm filter)
- Polaroid camera and color film to record results (optional)
- Automated microtiter plate washer or squirt bottle
- Vortex mixer
- Timer

ASSAY PROCEDURE

1 DNA EXTRACTION

1. **Dispense** 2 µL template DNA (from an initial concentration of about 150 ng/µL) to a thermoplate well or tube.

2. **Prepare** a master mix in a sterile vial, according to the volumes indicated in the table below, plus one spare reaction volume. Add the Taq DNA Polymerase to the amplification mix shortly before dispensing the mix. Mix gently by pipetting.

Master mix

Solution	Volume for one sample
Amplification Mix AAT	13.0 µL
Taq DNA Polymerase * (5 u/µL)	0.2 µL

*Not supplied.

The following Taq DNA polymerases were validated for use with this procedure (lacking 3' → 5' exonuclease activity):

- PHARMACIA Cat. No. 27-0799
- SIGMA Cat. No. D-1806
- ROCHE Cat. No. 1-146-165
- PROMEGA Cat. No. M-1661
- BIOLINE Cat. No. M95801B
- PERKIN ELMER Cat. No. M801-0060
- BIO LABS Cat. No. M2676

3. **Dispense** the total volume as indicated in the table above to each well or tube.
4. **Add** one drop of ColoRed™ Oil to each well. Do not touch the wells with the tip of the oil bottle. When using a thermocycler with a hot lid, it is not essential to use oil.
5. **Place** the thermoplate well or tube in a thermocycler previously programmed with the following protocol:

Cycling protocol

1.	96ºC	5 minutes	
2.	96ºC	15 seconds	35 cycles
3.	60ºC	15 seconds	
4.	72ºC	15 seconds	
5.	72ºC	5 minutes	

6. To verify amplification, **subject** 5 µL of the amplified product to electrophoresis in a 2% agarose gel.

Allele	Fragment size
PiS	202 bp
PiZ	243 bp

2 POST- AMPLIFICATION TREATMENT

Treat each 15 µL amplified DNA sample as follows:
5 µL - will be used to carry on this assay.
5 µL - are intended for retesting positive sample.
5 µL - recommended to be used for visualization of the amplified DNA by agarose gel electrophoresis.

1 **Prepare** a post-amplification treatment mix shortly before use. Combine in a single test tube the volumes appearing in the following table, multiplied by the number of tested samples, plus one spare volume.

Volumes for the post-amplification treatment

Solution	Volume for one sample
PRONTO® Buffer 2	25.0 µL
Solution C	1.0 µL
Solution D	0.75 µL

2 **Mix** gently by pipetting this solution in and out five times. Do not vortex.

3 **Add** 26 µL of the post-amplification mix into each well or tube containing 5 µL of each amplified DNA sample.
Ensure that the solution you add becomes well mixed with the DNA sample by inserting the tip under the oil, down to the bottom of the tube and mix the two solutions by pipetting.

4 **Add** one drop of ColoRed™ oil to each tube. Do not touch the tube with the tip of the oil bottle. When using a thermocycler with a hot lid, it is not essential to use oil.

5 **Incubate** for 30 minutes at 37ºC, then for 10 minutes at 95ºC in a thermocycler.
If not used immediately, the treated samples can be kept at 2-8ºC for a maximum of four hours.

3 PRIMER EXTENSION REACTION

1 **Program** the thermocycler as follows:

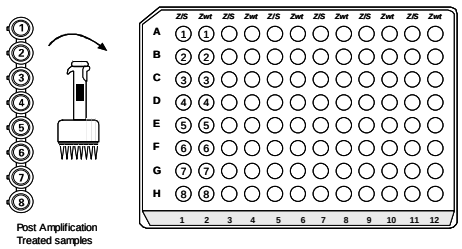
Cycle		Temperature	Time
Start:		96ºC	15 sec.
20 cycles:	{	96ºC	10 sec.
		65ºC	30 sec.
End:		18-25ºC - Cool down to room temperature	

2 **Take** a ProntoPlex™ Plate out of its pouch. Notice the color at the bottom of the wells. For each sample tested, use one pink well (*S/Z*) and one blue well (*Zwt*). Mark the plate with the ID numbers of your samples.

If you intend to use less than a full plate, you can cut the plate and return the unused portion to the pouch. If you do this, seal the pouch immediately with its desiccant card inside.

3 **Dispense** 8 µL of post-amplification treated DNA into the first two wells in row A (see Fig. 1). Continue with the remaining samples. It is possible to transfer up to eight samples simultaneously using a multichannel pipette. Ensure that the solution is at the bottom of each well by inspecting the plate from below. Make sure that the well does not contain air bubbles.

Figure 1: Scheme for dispensing post-amplification treated DNA samples into the ProntoPlex™ Plate



Recommendation:
Use a new set of tips for each column. Alternatively use the same set of tips, but do not touch the bottom of the wells.

4 **Tilt** the plate and add one drop of ColoRed™ oil to each well. Do not touch the well with the tip of the oil bottle*. When using a thermocycler with a hot lid, it is not essential to use oil.*
5 **Turn on** the thermocycler and start the cycling protocol.
6 When the thermal cycling is complete, you can proceed to the ELISA assay, or store the reaction products in the refrigerator and carry out the ELISA steps within 24 hours.

4 ELISA ASSAY - COLOR DEVELOPMENT

The ELISA assay consists of the following steps:
- **Binding** the biotinylated primer to the Streptavidin-coated plate.
- **Washing** away the unbound primer.
- **Incubating** with the HRP conjugate.
- **Washing** away the unbound conjugate.
- **Incubating** with the TMB Substrate (color development).

The results of this assay can be determined in one of two ways:

a **Visually**: by monitoring the development of the blue color.

or

b **Colorimetrically:** by adding Stop Solution and measuring the absorbance using an ELISA reader at a wavelength of 450 nm (yellow color).

> **! Before proceeding with the ELISA assay make your choice of visual or colorimetric determination of results.**

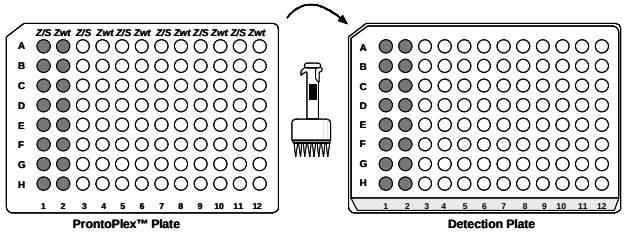
PREPARATION

- All components used in the detection step should reach room temperature before starting the assay.
- **Dilute** the 20x Wash Solution to 1x with deionized water.
  Dilute solution may be kept at 18-25º C for up to one month.
- **Peel** off the plastic cover of the Detection Plate. Mark the side of the plate with the kit name and test number.
- **Place** the PRONTO® plate and the Detection plate side by side, oriented in the same direction (see Fig. 2).

TRANSFER TO THE DETECTION PLATE

1 **Fill** a reagent reservoir /trough with the green colored Assay Solution. About 11 mL will be required for a 96-well plate.
2 **Add** 100 µL of Assay Solution to the bottom of each well in column 1 of the ProntoPlex™ Plate with a multichannel pipette. Mix the Assay Solution with the solution in the wells.
3 **Without changing tips, transfer** 100 µL from each well in this column to the first column in the Detection Plate (see Fig.2).
Ensure that the solution at the bottom of all wells of the ProntoPlex™ plate has turned green by inspecting them from below.

Figure 2: **Transferring the primer extension products from the ProntoPlex™ AAT Plate to detection plate.**



4 **Repeat** this procedure, using a new set of tips for each column. It is essential to maintain the order of the samples.
10 µL of oil carried over or 10 µL of the sample left behind will not significantly affect the detection process.

5 **Incubate** for 10 minutes at room temperature (18-25ºC).

DETECTION BY ELISA

Procedure	Visual Detection (Blue color)	Colorimetric Detection (Yellow color)
6 While the incubation with Assay Solution is taking place, **dilute** the Conjugated HRP in Assay Solution . For every detection plate used (96 wells), about 11 mL of diluted conjugate is required. **This solution should be freshly prepared each time the test is run.**	Dilution: 1:100 (110 µL of conjugated HRP into 11 mL Assay Solution)	Dilution: 1:300 (36 µL of conjugated HRP into 11 mL Assay Solution)
7 **Empty** the plate and wash four times with 350 µL 1x Wash Solution. Ensure that the plate is relatively dry after the last wash step.	√	√
8 **Add** 100 µL of freshly diluted conjugated HRP to all the wells, with a multichannel pipette.	√	√
9 **Incubate** at room temperature.	10 minutes	10 minutes
10 **Wash** the plate as in step 7.	√	√
11 **Add** 100 µL TMB-Substrate to each well with a multichannel pipette and incubate at room temperature (18-25ºC) until blue color appears	15 minutes	15 minutes
12 **Add** 100 µL of Stop Solution to each well with a multichannel pipette. The solution will turn yellow immediately.	—	100 µL
13 The results can be documented using a Polaroid camera with color film (for example - Fuji FP-100C), or by reading the absorbance using an ELISA reader (signal wavelength setting).	Agitate the plate gently and read results at O.D. 620 nm	—
14 Within two hours **read** the absorbance using an ELISA reader (single wavelength setting)	—	O.D. 450 nm

VALIDATION OF RESULTS

For Visual Detection:
For every sample tested, at least one of the two wells should develop a deep blue color. Otherwise, the results are invalid. Results should be interpreted as depicted in Fig. 3.

For Colorimetric Detection:
For every sample tested, at least one of the two wells should yield an O.D. ≥ 0.50 reading.

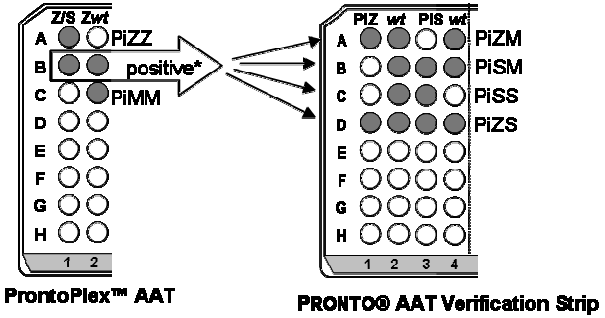
INTERPRETATION OF RESULTS

Important: Heterozygous or homozygous mutant results should be confirmed by retesting. It is recommended to repeat the test with newly extracted DNA.

Criteria for Visual Interpretation

A deep blue color indicates a positive signal, while negative signals appear as a clear or pale blue-colored well (see Fig. 3).

Figure 3: Visual Interpretation of Genotypes



Criteria for Colorimetric Interpretation

An O.D $_{450}$ value greater than 0.5 indicates a positive signal, while an O.D $_{450}$ value less than 0.35 indicates a negative signal.

Identify the correct genotype using the table below:

Genotype	*S/Z* well (O.D. 450)	*Z wt* well (O.D. 450)
Normal	O.D. ≤ 0.35	O.D. ≥ 0.5
AAT Positive*	O.D. ≥ 0.5	O.D. ≥ 0.5
PiZ Homozygote	O.D. ≥ 0.5	O.D. ≤ 0.35

! Samples with values not included in the above table are considered indeterminate and should be retested.

***** Positive samples may carry one of the following four genotypes: **PiMZ, PiMS, PiSS or PiSZ**
For the complete genotype determination use the PRONTO® AAT Verification Strip and follow the instructions starting at page 15.

ASSAY PROCEDURE – AAT VERIFICATION STRIP

! Each verification strip is sufficient for the full genotyping of two samples

1 POST-AMPLIFICATION TREATMENT

1 **Prepare** a post-amplification treatment mix shortly before use. Combine in a single test tube the volumes appearing in the following table, multiplied by the number of tested samples, plus one spare volume.

Volumes for the post-amplification treatment

Solution	Volume for one sample
PRONTO® Buffer 2	50.0 µL
Solution C	2.0 µL
Solution D	1.5 µL

2 **Mix** gently by pipetting this solution in and out five times. Do not vortex.

3 **Add** 53 µL of the Post Amplification mix into each well or tube containing 5 µL of each amplified DNA sample.
Ensure that the solution you add becomes well mixed with the DNA sample by inserting the tip under the oil, down to the bottom of the tube and mix the two solutions by pipetting.

4 **Add** one drop of ColoRed™ oil to each tube. Do not touch the tube with the tip of the oil bottle. *When using a thermocycler with a hot lid, it is not essential to use oil.*

5 **Incubate** for 30 minutes at 37ºC, then for 10 minutes at 95ºC in a thermocycler. If not used immediately, the treated samples can be kept at 2-8ºC for a maximum of four hours.

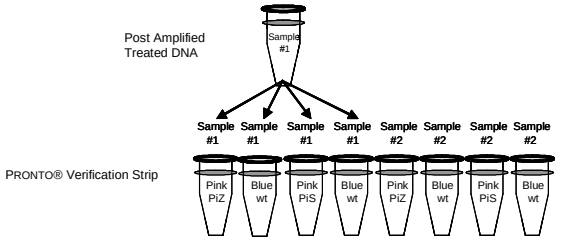
2 PRIMER EXTENSION REACTION

1 **Program** the thermocycler as in page 8.

2 **Take** a PRONTO® Verification Strip out of its pouch. Notice the color at the bottom of the wells. For each mutation tested, use one pink well (*mut*) and one blue well (wt). Mark the stripe with the ID numbers of your test.
If you intend to use less than a full strip, you can cut the strip and return the unused portion to the pouch. If you do this, seal the pouch immediately with its desiccant card inside.

3 **Dispense** 8 µL of each post-amplification treated DNA into four wells as shown in Fig. 4**.**

Figure 4: Scheme for dispensing post-amplification treated DNA samples into the PRONTO® AAT Verification strip:



Recommendation: Use a new set of tips for each well. Alternatively use the same set of tips, but do not touch the bottom of the wells.

4 **Add** one drop of ColoRed™ oil to each well.

5 **Turn on** the thermocycler and start the cycling protocol (page 8).

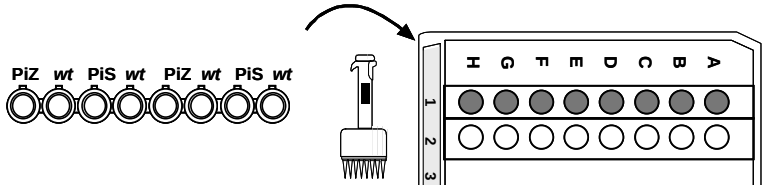
6 When the thermal cycling is complete, you can proceed to the ELISA assay, or store the reaction products in the refrigerator and carry out the ELISA steps within 24 hours.

TRANSFER TO THE DETECTION STRIP

1 **Take** the Detection strip out of its pouch.

2 **Place** a Detection strip in the detection plate frame and follow the instructions on page 10 (See Fig. 5).

Figure 5: Transferring the primer extension products from the PRONTO® AAT Verification Strip to the Detection Strip.



PRONTO® AAT Verification Strip Detection Strip

DETECTION BY ELISA

Procedure	Visual Detection (Blue color)	Colorimetric Detection (Yellow color)
6. While the incubation with Assay Solution is taking place, dilute the Conjugated HRP in Assay Solution . For every detection strip used (8 well), about 1 mL of diluted conjugate is required. **This solution should be freshly prepared each time the test is run.**	Dilution: 1:100 (10 µL of conjugated HRP into 1 mL Assay Solution)	Dilution: 1:300 (3.3 µL of conjugated HRP into 1 mL Assay Solution)
7-14 . Follow the instructions in the table on page 10.		

VALIDATION OF THE RESULTS

For Visual Detection:
For every mutation site tested, at least one of the wells should develop a deep blue color. Otherwise, the results are invalid for the relevant mutation.

For Colorimetric Detection:
For every mutation site tested, at least one of the two wells should yield an O.D. ≥ 0.50 reading.

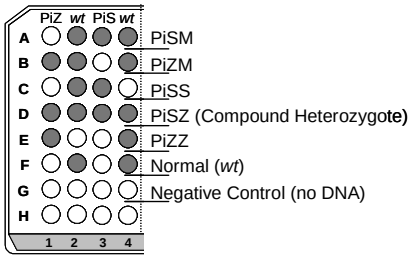
INTERPRETATION OF RESULTS

Important: Heterozygous or homozygous mutant results should be confirmed by retesting. It is recommended to repeat the test with newly extracted DNA.

Criteria for Visual Interpretation

A deep blue color indicates a positive signal, while negative signals appear as a clear or pale blue-colored well (see Fig. 6).

Figure 6: Genotype assignment according to visual inspection of test results



Criteria for Colorimetric Interpretation

The genotype of each sample is determined according to two criteria:
1. The O.D. values of the *mut* and *wt* wells.
2. The ratio of *mut / wt* O.D. values.

Calculate the *mut / wt* ratios by dividing the signal of the *mut* well by the signal of the *wt* well.
Identify the correct genotype using the table below:

Genotype	***mut* well** (O.D. 450)	***wt* well** (O.D. 450)	**mut/wt ratio**
Normal	O.D. ≤ 0.35	O.D. ≥ 0.5	< 0.5
Heterozygote	O.D. ≥ 0.5	O.D. ≥ 0.5	0.5 – 2.0
Homozygote	O.D. ≥ 0.5	O.D. ≤ 0.35	> 2.0

! Samples with values not included in the above table are considered indeterminate and should be retested.

ProntoPlex™ AAT - PROCEDURE SUMMARY

DNA EXTRACTION: from human whole blood, using a validated method.

DNA AMPLIFICATION: : **Volumes per reaction:** 2 µL template DNA + 13 µL Amplification Mix + 0.2 µL Taq Polymerase.
Cycling protocol:
96ºC 5 min→35 cycles of {96ºC 15 sec / 60ºC 15 sec / 72ºC 15 sec}→72ºC 5 min

POST AMPLIFICATION PROCEDURE:

- Volumes per reaction:

	ProntoPlex™ AAT	Pronto® AAT Verification Strip
PRONTO® Buffer 2	25.0 µL	50.0 µL
Solution C	1.0 µL	2.0 µL
Solution D	0.75 µL	1.5 µL

- **Pipette** in and out to mix.
- **Add** 26 µL into each well containing 5 µL amplified product (or 53 µL for the Verification Strip). Mix well.
- **Add** one drop of ColoRed™ oil.
- **Incubate** 30 minutes at 37ºC, then 10 minutes at 95°C.

PRIMER EXTENSION REACTION:

- **Dispense** 8 µL of each post-amplification treated DNA into two wells of the ProntoPlex™ plate (or four wells of the Verification Strip) .
- **Add** one drop of ColoRed™ oil.
- **Start** the cycling protocol:

96ºC 15 sec→20 cycles of {96ºC 10 sec. / 65ºC 30 sec.} →Cool.

DETECTION:

- **Add** 100 µL Assay Solution to each well in the PRONTOPlex™ Plate (or strip) and mix.
- **Transfer** 100 µL from each well of the ProntoPlex™ plate (or Verification Strip) to the identical position in the Detection Plate (or strip). Incubate 10 minutes at RT.
- **Empty** the wells and wash four times with 350 µL of 1x Wash Solution.

	Visual Detection	**Colorimetric Detection**
Add 100 µL of Conjugated HRP to every well and incubate for 10 minutes at RT.	Dilution 1:100	Dilution 1:300
Empty the wells and wash four imes with 350 µL of 1x Wash Solution.	√	√
Add 100 µL of TMB Substrate to each well and **Incubate** at RT for:	15 minutes	15 minutes
Add Stop Solution	—	100 µL per well
Read O.D. at:	620 nm	450 nm

For troubleshooting guide, please refer to our website:
www.prontodiagnostics.com/ts

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The Pronto® Technology is covered by US patent 5,710,028, by European patent 0648222 and by corresponding national patents.

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