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# Investigator<sup>®</sup> Validation Guide

For validation of Investigator STR PCR Kits in  
human identification and forensic applications

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# Contents

|   |    |
|---|----|
| Product Use Limitations .....   | 4  |
| Introduction .....  | 5  |
| About this guide .....  | 5  |
| Validations in human genetic identity and forensics laboratories .....                                  | 6  |
| Simplifying validation .....  | 7  |
| Definitions of Performance Data .....   | 8  |
| Reproducibility .....   | 8  |
| Sensitivity, limits of detection and stochastic effects .....   | 8  |
| Inhibition .....  | 9  |
| Degradation .....   | 9  |
| Concordance .....   | 10 |
| Cross-contamination .....   | 10 |
| DNA-mixtures .....  | 11 |
| Population data .....   | 11 |
| Technical instructions for validation of Investigator STR Kits using extracted<br>DNA as a sample ..... | 12 |
| Getting started .....   | 12 |
| Technical instructions .....  | 12 |
| Reproducibility .....   | 13 |
| Sensitivity, limits of detection and stochastic effects .....   | 14 |
| Inhibition studies .....  | 16 |
| Degradation studies .....   | 18 |

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|   |    |
|---|----|
| Concordance testing .....   | 19 |
| Cross-contamination.....  | 21 |
| Mixture studies .....   | 22 |
| Population studies.....   | 24 |
| Technical instructions for validation of Investigator STR GO! Kits for direct amplification ... | 25 |
| Important Notes .....   | 25 |
| Technical instructions .....  | 25 |
| Reproducibility .....   | 26 |
| Sensitivity, limit of detection and stochastic effects .....                                    | 27 |
| Concordance testing.....  | 29 |
| Cross-contamination.....  | 30 |
| Population studies.....   | 31 |
| References .....  | 33 |
| Ordering Information .....  | 34 |

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## Product Use Limitations

Investigator STR PCR Kits are intended for molecular biology applications in forensic, human identity, and paternity testing. These products are not intended for the diagnosis, prevention, or treatment of a disease.

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# Introduction

## About this guide

QIAGEN's Validation Guides assist with the design and practical experimental execution of a meaningful and representative validation plan, while considering time and other resource cost factors. This guide leads you through the validation of the Investigator STR PCR Kits, specifically, for the purpose of human identity and forensic applications. Within "Instructions for the experiments of the validation study", the guide covers validation procedures for extracted samples and direct amplification of reference samples. Please choose the part relevant for your application to be validated.

The guideline is divided into 5 parts:

- Introduction
- Definitions of performance data
- Instructions for the experiments of the validation study
- References
- Ordering information

A number of international and national authorities provide guidelines and recommendations for validation testing in human identity and forensic environments. Whenever applicable to short tandem repeat (STR) analysis, they were incorporated into this Validation Guide. These include recommendations of the European Network of Forensic Science Institutes (ENFSI) (1) and the Revised Validation Guidelines of the Scientific Working Group on DNA Analysis Methods (SWGDM) (2). For validation in the US, compliance with the latest revision of the Federal Bureau of Investigation (FBI)'s Quality Assurance Standards (QAS) is required for Forensic DNA Testing Laboratories.

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All QIAGEN technical and scientific literature is available at [www.qiagen.com](http://www.qiagen.com). Refer to the web site for the most current version of the Validation Guide.

Please note that this document is a proposal of QIAGEN GmbH and does not replace consultation with your competent authority to approve the necessary individual validation steps in your laboratory.

## Validations in human genetic identity and forensics laboratories

Validation of new equipment or methods is an important part of a forensic quality assurance system. When introducing a new technique to a laboratory, or when replacing an existing system or technique with a new one, it must be demonstrated that the new laboratory procedure is robust, reliable and reproducible when performed by the personnel in that laboratory (3).

A validation assesses analytical performance characteristics, such as precision and reproducibility, sensitivity and linearity. If the new method replaces, parallels or expands an existing procedure, a concordance study should also be included. Demonstrating the absence of cross-contamination events may also be considered. It is understood that known and non-probative evidence samples are used for validation testing (1).

The laboratory head is responsible for validation and release, after which the new technique can be used routinely for the generation of probative data.

The scope required for a validation depends on the specific equipment or method to be introduced in the laboratory. For example, does the new method apply to casework or database samples, or both? Has the new method previously demonstrated feasibility in a related context and therefore, does it require only supplementary study data?

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During the course of a validation study we recommend using known and non-probative evidence samples or mock evidence samples in accordance with validation guidelines in your region.

## Simplifying validation

There is considerable uncertainty among many laboratories about how a validation study should be set up. Variables include sample numbers, duration of study, and allocation of other resources.

Molecular forensics necessitates analysis of a multitude of sample types, varying greatly with respect to source, carrier or matrix, DNA quality and quantity, presence of inhibitors, or age. The validation must be conducted in agreement with guidelines or regulations issued by appropriate international or local authorities. Consequently, designing appropriate validation plans may appear challenging, with daunting experimental complexity and time requirements; it should not be.

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# Definitions of Performance Data

## Reproducibility

Reproducibility is defined as the ability of a system to generate the same data given the same input. "A subjective test method can be said to be valid if an examiner repeatedly obtains correct results for positive and negative known tests" (1).

In the case of STR analysis, this can be considered from two aspects. First, the PCR amplification must be capable of generating the same allelic calls when a sample is run multiple times; secondly, the system must generate allelic bands of the same peak height amplitude (within a tolerance and allowing for variation as a result of differences between capillary electrophoresis [CE] instruments).

## Sensitivity, limits of detection and stochastic effects

Sensitivity is defined as the ability of a system to yield correct and reproducible results with a given input. The limit of detection defines the point at which the sensitivity of the system is insufficient to detect an allele although it may be present in the sample." When appropriate, the range of DNA quantities able to produce reliable typing results should be determined" (4).

Stochastic effects may be observed in STR analysis where a sample contains very low amounts of DNA. In such cases, multiple aliquots of the sample will contain varying copies of the target DNA molecule. Stochastic effects will be observed if too small a quantity of DNA is amplified leading to allele drop-out and differential amplification of heterozygous alleles.



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## Inhibition

The PCR reaction may be inhibited when the extraction technique employed fails to remove extraneous materials from the sample. For this reason, we suggest the use of QIAGEN silica-bead or silica-column technology to generate DNA extracts free from inhibitory materials.

Extraneous materials commonly found in forensic samples arise from contact with the environment, such as humic compounds from soil and textile dyes from fabric such as denim. In addition, some sample types also naturally contain substances that inhibit PCR, such as hematin in blood and calcium ions in bone.

Given the wide range of PCR-inhibiting molecules and the difficulties associated with some extraction techniques in removing these molecules, it is critical that the PCR reaction offers resistance to inhibition.

## Degradation

Degradation studies are of critical importance to forensic applications; especially considering the nature of some sample types that may be subject to natural degradation in the environment before sampling and appropriate storage. Degradation is a natural component of cell death and cell decomposition. During that process, enzymes are released by the cell as it decays. These enzymes break down the chromosomal DNA, reducing it to increasingly smaller fragment sizes.

STR kits must offer a level of resistance to degradation in order to obtain valuable data from such samples. Resistance is generally achieved with the use of mini- and midi-sized STR fragments that can be successfully amplified, even considering the small size of the flanking region to which the PCR primer will anneal and of the amplicon itself.

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## Concordance

In the case of STR analysis, concordance questions whether a sample with a known DNA profile will have the same DNA profile when examined using a different PCR amplification system. PCR amplification systems use PCR primers designed to anneal to STR target sites flanking regions known to be stable amongst many populations. However, when examining large sample numbers, variations in the flanking region can be expected. The variations may lead to either differences in the length of the amplified sequence or, in the worst case, failure to amplify the sequence leading to allele dropout.

Laboratories currently performing STR analysis must evaluate the level of concordance new PCR systems offer against the system currently in use. As the level of concordance is expected to be very high, this may involve the analysis of many hundreds or thousands of samples. As such, we recommend that laboratories obtain published concordance data from organizations such as the National Institute of Standards and Technology (NIST) or the ENFSI. Some guidance for conducting a concordance study is provided in this document.

## Cross-contamination

Cross-contamination is of significant importance in the forensic environment. Cross-contamination may arise from sample handling issues during sample collection at the crime scene or via sample handling errors or pipetting errors at the processing laboratory. Cross-contamination may lead to incorrect assessments of samples and sample mixtures.

A contamination study is relevant to any new DNA STR profiling system being employed, to ascertain if the processes leading up to the generation of the STR profile are able to be performed without resulting in cross-contamination. The Investigator STR PCR Kits are highly sensitive and may be more susceptible to cross-contamination. Such events may previously have been undetected and tighter controls may be required within the laboratory to ensure that cross-contamination events do not occur.

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## DNA-mixtures

Laboratories performing STR analysis from samples from crime scenes samples must demonstrate “the ability to obtain reliable results from mixed source samples” (4). A common attribute of crime scene samples is the limited amount of DNA contained within the samples. As heterozygote balance is known to be adversely affected in all PCR systems at low DNA concentrations, it is important to determine the point where heterozygote balance becomes so poor that the analysis of a mixture becomes impractical or leads to the incorrect determination of the mixture. In addition, due to the limitations of PCR at very low DNA concentrations, alleles may not be observed within the electropherogram as a result of stochastic effects.

## Population data

In order to assign weight of evidence to a profile generated, it is necessary to understand the frequency by which each allele within the profile is observed in the population. To enable this, a statistically significant number of individuals from the relevant population must be examined. Generally, it is accepted as significant when no less than 200 individuals have been examined, although this may differ for your country or in highly polymorphic populations. The figure of 200 individuals is supplied only as guidance.

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# Technical instructions for validation of Investigator STR PCR Kits using extracted DNA as a sample

## Getting started

Refer to the *Investigator STR PCR Kit Handbook* relevant to the study being performed for detailed information on kit content, protocols and materials required, but not included (e.g., matrix for spectral calibration, general PCR and capillary electrophoresis equipment). Ensure all materials required to perform this study are available before starting.

## Technical instructions

The following pages contain details of experimental setups as a guide to assist you in the testing and validation of the QIAGEN Investigator STR PCR Kits. You should examine the guidance and determine whether they are sufficient for a validation study to meet the standards that apply in your country, and perform further studies, if required.

When performing validation studies:

- Set up all reaction mixtures in an area separate from that used for DNA isolation, PCR amplification, and PCR product analysis (post-PCR).
- Use disposable tips containing hydrophobic filters to minimize cross-contamination.
- Before opening the tubes with PCR components, vortex and then centrifuge briefly to collect contents at the bottom of the tubes.

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## Reproducibility

### Important point before starting

- In order to eliminate variations in peak height data, where possible, use the same Applied Biosystems® Genetic Analyzer CE instrument used for the analysis of the STR data.

### Procedure

1. Obtain 20–30 samples and accurately determine the DNA concentration of each using real-time PCR.
2. Amplify each sample in triplicate. Samples may be named using the following nomenclature: “sample name\_triplicate\_x” where x is the repeat number of the triplicate, i.e., 1, 2 or 3.

Run 1: Perform the CE run using the parameters described in the relevant *Investigator STR PCR Kit Handbook*.

### Data analysis: Assessing allele calling reproducibility

1. Examine each of the samples in the run using GeneMapper® ID Software, GeneMapper ID-X Software, or other analysis software validated in your laboratory, removing any non-allelic peaks.
2. Compare the allele calls of each sample in the triplicate. Examine the whole profile to ensure concordant data has been generated for each sample.

### Data analysis: Peak height reproducibility – part 1

#### Analysis using GeneMapper ID or GeneMapper ID-X Software

1. Open GeneMapper Manager, select the plot settings tab, and then open the plot settings.
2. Select the labels tab, change label 2 to height, and click “OK” to save the change.

3. With GeneMapper manager open, select the table settings tab, then open the “Data Analysis” table setting.
4. Select the Genotypes tab and check the box on the Height column. Using the data analyzed, sort the data by sample name, and then export the data from the genotypes table to a text file.

If you are using a different analysis platform, consult the user manual for that software to determine how to change the data to be exported.

### Data analysis: Peak height reproducibility – part 2

1. Open the exported data file in Microsoft® Excel®, perform a comparison of the peak heights at each locus, for each of the samples in the triplicate.
2. Using Excel, determine the standard deviation for the peak heights and plot this data, if required.

## Sensitivity, limits of detection and stochastic effects

### Important points before starting

- The sensitivity and the limits of detection will vary between Applied Biosystems Genetic Analyzer CE instruments. If your laboratory has access to multiple instruments, we recommend you identify the variation between these instruments before starting the study. We also recommend that the study be performed using one analysis instrument. If the instrument-to-instrument variation is found to be quite high, consider this when analyzing the data generated by the study.
- Stochastic effects are commonly observed when samples contain very low DNA concentrations, such that multiple aliquots of the same sample will result in the collection of different amounts of the target molecule from the sample and thus generate profiles where alleles may drop out. This effect must be examined so that the point at which stochastic effects become apparent is understood. Accordingly, the processing regime for

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samples of a concentration at or below this point may be adjusted to ensure consistent results are obtained.

- Accurate pipetting is required. We recommend calibrating all pipets used in this study prior to starting. Remedial action should be carried out, if required.
- The Investigator STR PCR Kit protocols provide the option of increasing the total PCR cycle number for low template samples. If this is required, the study should be performed using the higher cycle count in order to resolve alleles from low DNA template concentration samples.

### Things to do before starting

- Obtain samples with a known concentration above 1 ng/μl.
- Quantify the known DNA sample and normalize to 1 ng/μl. Re-quantify the normalized sample to ensure a target of 1 ng/μl has been achieved.

### Procedure

1. Perform serial dilutions of the known DNA sample. Label seven 0.5 ml tubes as follows:
  - Tube 1: 1 ng/μl
  - Tube 2: 500 pg/μl
  - Tube 3: 250 pg/μl
  - Tube 4: 125 pg/μl
  - Tube 5: 62.5 pg/μl
  - Tube 6: 31.3 pg/μl
  - Tube 7: 16 pg/μl
2. Pipet 100 μl Low-salt TE Buffer into tubes 2–7.
3. Pipet 200 μl of a known DNA sample normalized to 1 ng/μl into tube 1.
4. Transfer 100 μl from tube 1 to tube 2.
5. Seal the tube and vortex thoroughly.

6. Repeat steps 4 and 5 transferring diluted DNA to the next tube in turn, until the final tube contains DNA at a concentration of 16 pg/μl.
7. Set up PCR runs in triplicate for tubes 2–7, according to the protocol “PCR Amplification” in the relevant *Investigator STR PCR Kit Handbook*. Use 1 μl of each of the serial dilutions (tube 1 is not required). In addition, set up a tube containing TE buffer and PCR primer/master mix as a negative control, and label this as tube 8.

Run: Perform the first CE run using the parameters described in the relevant *Investigator STR PCR Kit Handbook*.

## Data analysis

1. Examine each of the samples in turn and identify the point at which the first alleles drop out. Once the sample has been identified, proceed to step 2.
2. Compare each of the 3 replicates for the identified sample to ensure that the allele dropout observed is consistent, ruling out any outliers where appropriate.
3. Identify the point at which alleles drop out. This determines the sensitivity of the test and also the stochastic threshold.
4. Identify the point at which the test fails to amplify alleles above the minimum callable threshold employed in your laboratory. This determines the limit of detection.

## Inhibition studies

### Things to do before starting

- Obtain a high-quality supply of humic acid (available from suppliers including Alfa Aesar), hematin (available from suppliers including ICN Biomedicals), calcium (calcium dihydrogen; phosphate available from suppliers including VWR International) and indigo (indigo carmine; available from suppliers including Alfa Aesar).
- Prepare stock solutions of each inhibitor as follows:
  - Humic acid (Alfa Aesar), 1 mg/ml in water



- Hematin: Dissolve at 25 mM in 0.1 N sodium hydroxide; dilute to 3.5 mM in water
- Calcium dihydrogen phosphate: 20 mM in water
- Indigo carmine (Alfa Aesar), 30 mM in water
- All subsequent dilutions should be prepared using water.
- Identify a panel of samples with concentrations around 1 ng/μl known to generate full profiles which do not exhibit any of the typical signs of inhibition (i.e., dropout of high molecular weight loci).

**Table 1. Inhibitor pipetting scheme**

| Inhibitor                    | Stock concentration | Final concentration | μl Inhibitor per 25 μl reaction |
|------------------------------|---------------------|---------------------|---------------------------------|
| Humic acid                   | 1000 ng/μl          | 50 ng/μl            | 1.3                             |
|                              |                     | 100 ng/μl           | 2.5                             |
|                              |                     | 150 ng/μl           | 3.8                             |
| Hematin                      | 3.5 mM              | 250 μM              | 1.8                             |
|                              |                     | 500 μM              | 3.6                             |
|                              |                     | 750 μM              | 5.4                             |
| Calcium dihydrogen phosphate | 20 mM               | 1 mM                | 1.3                             |
|                              |                     | 1.5 mM              | 1.9                             |
|                              |                     | 2 mM                | 2.5                             |
| Indigo carmine               | 20 mM               | 4 mM                | 3.3                             |
|                              |                     | 6 mM                | 5.0                             |
|                              |                     | 8 mM                | 6.7                             |

### Procedure: Humic acid

1. Create several 50 μl aliquots of the identified DNA samples.
  2. Set up PCR reactions according to the protocol “PCR Amplification” (see any *Investigator STR PCR Kit Handbook*) in triplicate, as follows.
- Pipet the target quantity of DNA into each of the replicates, as specified in the relevant *Investigator STR PCR Kit Handbook*.

- Add in the desired amount of inhibitor to obtain a final inhibitor concentration of 50 ng/μl, 100 ng/μl, and 150 ng/μl.
- Make up the final reaction volume using nuclease-free water.

Run: Perform the CE run using the parameters described in the relevant *Investigator STR PCR Kit Handbook*.

#### Procedure: Hematin

Repeat the procedure as shown for humic acid, substituting hematin for humic acid at concentrations of 250 μM, 500 μM and 750 μM.

#### Procedure: Calcium

Repeat the procedure as shown for humic acid, substituting calcium for humic acid at concentrations of 1 mM, 1.5 mM and 2 mM.

#### Procedure: Indigo

Repeat the procedure as shown for humic acid, substituting indigo for humic acid at concentrations of 4 mM, 6 mM and 8 mM.

#### Data analysis

Examine each of the samples in turn and identify the point at which inhibition of the PCR reaction can be identified.

### Degradation studies

#### Important point before starting

- Be sure to obtain a panel of samples that display a typical degradation pattern where low-molecular-weight fragments will be well amplified and the level of amplification diminishes as fragment size increases until alleles are no longer observed.

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## Procedure

1. Create several 50 µl aliquots of the identified samples.
2. Quantify the samples using real-time PCR.
3. Amplify the samples using the current STR chemistry.
4. Amplify the samples as per the protocol "PCR Amplification" (see any *Investigator STR PCR Kit Handbook*). Ensure the total DNA input is appropriate for the kit being used.

Run 1: Run the samples amplified using the current PCR chemistry employed by the laboratory, using the current CE method.

Run 2: Perform the CE run using the parameters described in the relevant *Investigator STR PCR Kit Handbook*.

## Data analysis

Compare the allele calls of each of the samples amplified using the current PCR technology with the samples amplified using the Investigator STR PCR Kit.

## Concordance testing

### Important points before starting

- Undertaking a concordance study requires a ready supply of samples that have either been profiled previously using the existing system or that are available in sufficient quantity to be analyzed in duplicate. Note that some samples may require further repetition to confirm non-concordance events with a view to possible sequencing of the locus in the identified sample (if facilities are available).
- Ensure the samples used in this study contain good-quality DNA, i.e., they are derived from recent extracts of buccal swab or blood samples that have been appropriately stored at -20°C since taking from the subject.

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## Procedure

1. Amplify the samples using the current STR chemistry.
2. Amplify the samples as per the protocol “PCR Amplification” (see any *Investigator STR PCR Kit Handbook*). Ensure the total DNA input is appropriate for the kit being used.

Run 1: Run the samples amplified using the current PCR chemistry employed by the laboratory, using the current CE method.

Run 2: Perform the CE run using the parameters described in the relevant *Investigator STR PCR Kit Handbook*.

## Data analysis

1. Examine each of the samples in the run using GeneMapper ID software, GeneMapper ID-X software, or other analysis software validated in your laboratory, removing any non-allelic peaks.
2. Compare the allele calls of each of the samples amplified using the current PCR technology with the samples amplified using the Investigator STR PCR Kit.
3. Identify any non-concordant samples.
4. Re-examine both amplifications of the sample to ensure allele calling is accurate. Ensure any non-concordant alleles are not due to allele dropout associated with low template amplification or degradation.
5. Any samples with identified concordance issues should be re-amplified and examined using the currently used STR chemistry and the Investigator STR PCR Kit to confirm the non-concordant event.
6. Calculate the frequency of concordance issues per locus using the following formula:

$$\text{Non-concordance} = \frac{\text{Number of non-concordant alleles}}{\text{Total number of alleles}}$$

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## Cross-contamination

### Important points before starting

- Ensure all equipment that will be used in the study has been decontaminated using the standard method employed by your laboratory.
- Cross-contamination events may occur at any point in the process; therefore, the entire process must be examined.
- Cross-contamination testing may be performed either in tubes or 96-well plates. The procedure below is the suggested method for performing a validation study using 96-well plates. This can be adapted for tubes by inserting the tubes into tube racks in a 96-well format.

### Procedure

1. Set up a 96-well plate with alternate samples and blank wells (checker board layout).
2. Perform DNA extraction and quantification following your normal laboratory procedure.
3. Amplify the entire 96-well plate. Ensure that the empty wells are amplified as negative controls (i.e., amplify the maximum permitted sample volume according to the Investigator STR PCR Kit being used).

Run: Perform the CE run using the parameters described in the relevant *Investigator STR PCR Kit Handbook*.

### Data analysis

1. Analyze the data using an analysis method suitable for detecting low amplitude alleles, i.e., <50 RFU.
2. Examine each of the blank samples for the presence of allelic bands.
3. If any contamination is observed, attempt to locate the sample from which the contaminating allele(s) have originated.

4. Any allelic bands identified should be examined to identify where in the process they were introduced. This may be done by re-electrophoresis of the sample in question. If the allele is still present, proceed to re-amplification, if the allele is still present after re-amplification, the contamination may have resulted from a transfer step at DNA extraction, quantification or PCR; however it is not possible to ascertain in which process this will have originated. As such, each process must be examined for sources of cross-contamination and amended, as required.

### Alternating blank columns and/or rows

The cross-contamination study may be repeated if required using a plate layout arranged with alternating blank rows and/or columns. This is particularly useful in an automated process where instruments may track across plates. If this is required, repeat the procedure specified in this section and place empty wells in either alternating rows or columns of the plate as required.

## Mixture studies

### Important point before starting

- Ensure samples used in this study are known to be from a single source, i.e., reference samples taken from known individuals.

### Procedure: Determining heterozygote balance

1. From the dilution series created in "Sensitivity and limit of detection" (page 15), take the data from the samples of concentrations 62.5 pg/μl, 125 pg/μl, and 250 pg/μl.
2. Analyze the peak heights for each locus, identifying the point at which the heterozygous balance falls below that which is required by your laboratory.
3. When exporting from GeneMapper ID or GeneMapper ID-X Software ensure that the "duplicate homozygotes" option is disabled.
4. Open the exported data in Excel and remove all homozygous alleles.

5. Calculate the heterozygous ratio using the following formula:

$$\text{Heterozygous ratio} = \frac{\text{Peak height of Allele 1}}{\text{Peak height of Allele 2}}$$

#### Procedure: Mixture analysis

1. Create mixtures using the following ratios (Sample A:Sample B):

|      |      |
|------|------|
| 1:0  | 0:1  |
| 1:2  | 2:1  |
| 1:5  | 5:1  |
| 1:10 | 10:1 |
| 1:20 | 20:1 |

2. Amplify the mixtures and run the PCR product on an Applied Biosystems Genetic Analyzer CE instrument, according to the handbook of the Investigator STR PCR Kit being used.

#### Data analysis

1. Analyze the mixture data using the mixture interpretation process validated in your laboratory.
2. Calculate the mixture ratio (Mx) for each of the mixtures and compare against the known mixture ratios.

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## Population studies

### Important points before starting

- Ensure individuals used in this study are unrelated.
- Samples used in the study may be used from pre-extracted samples or from fresh sample extracts.
- Process 10% more samples than required, to allow for samples which fail to yield a full profile.

### Procedure

1. Extract, quantify, and amplify >200 samples.
2. Analyze the samples using an Applied Biosystems Genetic Analyzer CE instrument.

### Data analysis

1. Analyze the data using an analysis method suitable for detecting well-amplified alleles, i.e., >150 RFU.
2. Remove any samples from the data set that contain failed loci.
3. Count the total number of samples yielding a full STR profile.
4. Determine the total number of called alleles for each locus in the population group.
5. Determine how often each of the observed alleles occurs in the population. Repeat for each locus.
6. Take the data from points 4 and 5 to determine the frequency of each allele in the population using the following equation:

$$\text{Allele frequency} = \frac{\text{Total observations of the allele}}{\text{Total allele count at the locus}}$$



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# Technical instructions for validation of Investigator STR GO! Kits for direct amplification

## Important Notes

The experimental conditions outlined in the Investigator STR GO! Kit protocols have been found to deliver the best results. However, depending on the sample material, PCR cycle numbers may be adapted to ensure the highest possible first-round success rates. We recommend running a representative batch of samples in order to confirm that the cycle numbers given in the protocols are optimal. Increase the cycle number by one if the signals in the resulting electropherograms are too low. Decrease the cycle number by one if the signals in the resulting electropherograms are too high. Adjustment of the cycle number should be performed before starting a validation study.

## Technical instructions

The following pages contain details of experimental setups as a guide to assist you in the testing and validation of the QIAGEN Investigator STR GO! Kits. Setups are suggested for each of the most common sample types: buccal swabs and blood or buccal cells on FTA<sup>®</sup> paper. Choose sample types for your study according to your routine use samples. You should examine the guidance and determine whether they are sufficient for a validation study to meet the standards that apply in your country, and perform further studies, if required.

When performing validation studies:

- Set up all reaction mixtures in an area separate from that used for DNA isolation, PCR amplification and PCR product analysis (post-PCR).
- Use disposable tips containing hydrophobic filters to minimize cross-contamination.

- Before opening the tubes with PCR components, vortex and then centrifuge briefly to collect contents at the bottom of the tubes.

## Reproducibility

### Important point before starting

- In order to eliminate variations in peak height data, where possible, use the same Applied Biosystems Genetic Analyzer CE instrument used for the analysis of the STR data.

### Procedure

1. Obtain 20–30 samples.
2. Amplify each sample in triplicate. Samples may be named using the following nomenclature: “sample name\_triplicate\_x” where x is the repeat number of the triplicate, i.e., 1, 2 or 3.

For buccal swabs, amplify triplicates from one swab lysate. For FTA cards, take 3 spots of 1.2 mm diameter from one card.

Run 1: Perform the CE run using the parameters described in the relevant *Investigator STR PCR Kit Handbook* (i.e., injection for 10 s at 3 kV).

### Data analysis: Assessing allele calling reproducibility

1. Examine each of the samples in the run using GeneMapper ID Software, GeneMapper ID-X Software, or other analysis software validated in your laboratory, removing any non-allelic peaks.
2. Compare the allele calls of each sample in the triplicate. Examine the whole profile to ensure concordant data has been generated for each sample.

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## Sensitivity, limit of detection and stochastic effects

### Important points before starting

- The sensitivity and the limit of detection will vary between Applied Biosystems Genetic Analyzer CE instruments. If your laboratory has access to multiple instruments, we recommend you identify the variation between these instruments before starting the study. We also recommend that the study be performed using one analysis instrument. If the instrument-to-instrument variation is found to be quite high, consider this when analyzing the data generated by the study.
- Stochastic effects are commonly observed when samples contain very low DNA concentrations, such that multiple aliquots of the same sample will result in the collection of different amounts of the target molecule from the sample and thus generate profiles where alleles may drop out. Although reference samples are expected to be of good quality, this effect must be examined so that the point at which stochastic effects become apparent is understood.
- Accurate pipetting is required. We recommend calibrating all pipets used in this study prior to starting. Remedial action should be carried out, if required.
- Choose sample types for your study according to your routine use samples. For buccal swabs, sensitivity can be addressed using dilutions of a buccal swab lysate. For blood or buccal cells on FTA, dilutions of blood or saliva are spotted on cards.

### Things to do before starting

- Obtain a buccal swab lysate, or a blood or saliva sample of >200 µl.

### Procedure

1. Perform serial dilutions of the sample. Label seven 0.5 ml tubes as follows:

Tube 1: undiluted

Tube 2: 1:4

Tube 3: 1:8

Tube 4: 1:16

Tube 5: 1:32

Tube 6: 1:64

Tube 7: 1:128

2. Pipet 100  $\mu$ l Low-salt TE Buffer into tubes 2–7.
3. Pipet 200  $\mu$ l of a sample into tube 1.
4. Transfer 100  $\mu$ l from tube 1 to tube 2.
5. Seal the tube and vortex thoroughly.
6. Repeat steps 4 and 5 transferring diluted DNA to the next tube in turn until the final tube contains DNA at a dilution of 1:128.

Dilutions of buccal swab lysates are now ready to be used. To prepare blood or saliva dilutions on FTA, spot 70  $\mu$ l of each dilution on a separate card and let dry overnight.

7. Set up PCR reactions in triplicate for tubes 2–7 according to the protocol “PCR Amplification” in the relevant *Investigator STR GO! Kit Handbook*. Use 2  $\mu$ l of each of the serial dilutions (buccal swab lysate), or a 1.2 mm punch taken from the center of the FTA card. In addition, set up a tube containing TE buffer and PCR primer/master mix as a negative control and label this as tube 8.

## Data analysis

1. Examine each of the samples in turn and identify the point at which the first alleles drop out. Once the sample has been identified, proceed to step 2.
2. Compare each of the 3 replicates for the identified sample to ensure that the allele dropout observed is consistent, ruling out any outliers where appropriate.
3. Identify the point at which alleles drop out. This determines the sensitivity of the test and also the stochastic threshold.
4. Identify the point at which the test fails to amplify alleles above the minimum callable threshold employed in your laboratory. This determines the limit of detection.

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## Concordance testing

### Important points before starting

- Undertaking a concordance study requires a ready supply of samples that have either been profiled previously, using the existing system, or that are available in sufficient quantity to be analyzed in duplicate. Note that some samples may require further repetition to confirm non-concordance events with a view to possible sequencing of the locus in the identified sample (if facilities are available).
- Ensure the samples used in this study are of good quality.

### Procedure

1. Amplify the samples using the current STR chemistry.
2. Amplify the samples as per the protocol “PCR Amplification” (see any *Investigator STR GO! Kit Handbook*).

### Data analysis

1. Examine each of the samples in the run using GeneMapper ID software, GeneMapper ID-X software or other analysis software validated in your laboratory, removing any non-allelic peaks.
2. Compare the allele calls of each of the samples amplified using the current PCR technology with the samples amplified using the Investigator STR GO! Kit.
3. Identify any non-concordant samples.
4. Re-examine both amplifications of the sample to ensure allele calling is accurate. Ensure any non-concordant alleles are not due to allele dropout associated with low template amplification or degradation.
5. Any samples with identified concordance issues should be re-amplified and examined using the currently used STR chemistry and the Investigator STR GO! Kit to confirm the non-concordant event.

6. Calculate the frequency of concordance issues per locus using the following formula:

$$\text{Non-concordance} = \frac{\text{Number of non-concordant alleles}}{\text{Total number of alleles}}$$

## Cross-contamination

### Important points before starting

- Ensure all equipment that will be used in the study has been decontaminated, using the standard method employed by your laboratory.
- Cross-contamination events may occur at any point in the process; therefore, the entire process must be examined.
- Cross-contamination testing may be performed either in tubes or 96-well plates. The procedure below is the suggested method for performing a validation study using 96-well plates. This can be adapted for tubes by inserting the tubes into tube racks in a 96-well format.
- Choose sample types for your study according to your routine use samples.

### Procedure

1. Set up a 96-well PCR plate with alternate samples and blank wells (checker board layout). Use 2 µl of Investigator STR GO! Lysis Buffer as negative control samples when testing buccal swab samples. Take 1.2 mm spots from empty cards as negative samples when testing blood or saliva on FTA and other paper.
2. Amplify the entire 96-well plate.

Run: Perform the CE run using the parameters described in the relevant *Investigator STR PCR Kit Handbook* (i.e., inject for 10 s at 3 kV).

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## Data analysis

1. Analyze the data using an analysis method suitable for detecting low amplitude alleles, i.e., <50 RFU.
2. Examine each of the blank samples for the presence of allelic bands.
3. If any contamination is observed, attempt to locate the sample from which the contaminating allele(s) have originated.
4. Any allelic bands identified should be examined to identify where in the process they were introduced. This may be done by re-electrophoresis of the sample in question. If the allele is still present, proceed to re-amplification.

## Alternating blank columns and/or rows

The cross-contamination study may be repeated if required using a plate layout arranged with alternating blank rows and/or columns. This is particularly useful in an automated process where instruments may track across plates. If this is required, repeat the procedure specified in this section and place empty wells in either alternating rows or columns of the plate as required.

## Population studies

### Important points before starting

- Ensure individuals used in this study are unrelated.
- Samples used in the study may be used from pre-extracted samples or from fresh sample extracts.
- Process 10% more samples than required, to allow for samples which fail to yield a full profile.

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## Procedure

1. Extract, quantify, and amplify >200 samples.
2. Analyze the samples using an Applied Biosystems Genetic Analyzer CE instrument.

## Data analysis

1. Analyze the data using an analysis method suitable for detecting well-amplified alleles, i.e., >150 RFU.
2. Remove any samples from the data set that contain failed loci.
3. Count the total number of samples yielding a full STR profile.
4. Determine the total number of called alleles for each locus in the population group.
5. Determine how often each of the observed alleles occurs in the population. Repeat for each locus.
6. Take the data from points 4 and 5 to determine the frequency of each allele in the population using the following equation:

$$\text{Allele frequency} = \frac{\text{Total observations of the allele}}{\text{Total allele count at the locus}}$$



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# References

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# Ordering Information

| Product                                       | Contents  | Cat. no. |
|---|---|----------|
| Investigator<br>24plex QS Kit (100)*†         | Primer mix, Fast Reaction Mix 2.0, Control DNA, allelic ladder 24plex, DNA size standard 550 (BTO), and nuclease free water       | 382415   |
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| Investigator<br>ESSplex Plus Kit (100)*†      | Primer mix, Fast Reaction Mix, Control DNA, allelic ladder ESSplex Plus, DNA size standard 550 (BTO) and nuclease-free water      | 381535   |
| Investigator<br>ESSplex SE QS Kit<br>(100)†   | Primer mix, Fast Reaction Mix 2.0, Control DNA, allelic ladder ESSplex SE QS, DNA size standard 550 (BTO) and nuclease free water | 381575   |
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| Investigator<br>IDplex Plus Kit (100)*†       | Primer mix, Fast Reaction Mix, Control DNA, allelic ladder IDplex Plus Plus, DNA size standard 550 (BTO) and nuclease-free water  | 381625   |
| Investigator<br>IDplex GO! Kit (200)*†        | Primer Mix, Fast Reaction Mix, Control DNA, allelic ladder IDplex GO! and DNA size standard 550 (BTO)                             | 381636   |

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| <b>Product</b>                               | <b>Contents</b>   | <b>Cat. no.</b> |
|--|---|-----------------|
| Investigator<br>Triplex DSF Kit (400)*       | Primer mix, reaction mix, DNA Polymerase,<br>Control DNA, allelic ladder, DNA size standard<br>and nuclease-free water                  | 380327          |
| Investigator<br>Triplex AFS QS Kit<br>(400)* | Primer mix, reaction mix, DNA Polymerase,<br>Control DNA, allelic ladder, DNA size standard<br>and nuclease-free water                  | 380317          |
| Investigator<br>HDplex Kit (100)*            | Primer mix, reaction mix, DNA Polymerase,<br>Control DNA, allelic ladder, DNA size standard<br>and nuclease-free water                  | 381215          |
| Investigator<br>Argus X-12 QS Kit (25)†      | Primer mix, Fast Reaction Mix 2.0, Control DNA,<br>allelic ladder Argus X-12 QS, DNA size standard<br>550 (BTO) and nuclease free water | 383223          |
| Investigator<br>Argus Y-12 QS Kit<br>(100)*  | Primer mix, reaction mix, DNA Polymerase,<br>Control DNA, allelic ladder, DNA size standard<br>and nuclease-free water                  | 383615          |
| Investigator<br>DIPlex Kit (100)             | Primer mix, reaction mix, DNA Polymerase,<br>Control DNA, allelic ladder, DNA size standard<br>and nuclease-free water                  | 384015          |

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