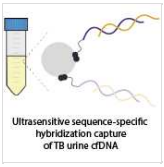

Feb 27, 2021

Ultrasensitive hybridization capture of short tuberculosis cell-free DNA from urine

PLOS One

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1 Works for me dx.doi.org/10.17504/protocols.io.bep4jdqw

Amy Oreskovic

SUBMIT TO PLOS ONE

ABSTRACT

Overview: This protocol describes a method for highly sensitive sequence-specific purification of short tuberculosis (TB) urine cell-free DNA (cfDNA) from large-volume (10 mL) urine samples. Biotinylated oligonucleotide capture probes complementary to the target of interest are immobilized on streptavidin-coated magnetic beads and used to capture, concentrate, and purify target DNA via hybridization. This protocol improves upon the analytical performance of both existing silica-based extraction methods for urine cfDNA and previous hybridization capture protocols, and meets several previously unmet design criteria for urine cfDNA: (1) **high recovery of short fragments**, (2) **large sample input volume**, and (3) **<1 copy/mL sensitivity**. There are two key innovations that contribute to the robustness and unprecedented sensitivity of this method: **dual biotinylated capture probes**, which increase recovery compared to single biotinylated probes by moderating probe density on the bead surface (a key variable affecting efficiency of surface-based hybridization), improving thermostability of the bead-probe linkage, and eliminating interference by endogenous biotin in urine, and a **two-probe system** for each target region, which enables recovery of both strands of double-stranded DNA. We designed this hybridization method for capture of short, dilute TB cfDNA fragments from urine, but anticipate that it will be versatile and may be useful for other applications and sample types requiring sensitive and efficient purification of DNA from large sample volumes. Design of primer and probe sequences for new targets is straightforward (see "Materials" tab).

Expected performance:

- Near 100% recovery (95% CI: 82.6 - 117.6%) of synthetic ssDNA or dsDNA spiked into 10 mL urine samples, verified across concentrations of at least 1 – 10,000 copies/mL and fragment lengths of at least 25 – 150 bp
- Limit of detection of ≤5 copies of dsDNA in 10 mL urine (0.5 copies/mL)
- Enables amplification of DNA from 10 mL urine in a single PCR well (500X concentration factor)
- Tolerant to variations in sample composition, including pH (5 – 8), salt (0 – 500 mM), and non-target DNA (0 – 10 µg)

Graphic abstract: (A) Schematic of capture probe design. **(B)** Overview of workflow.

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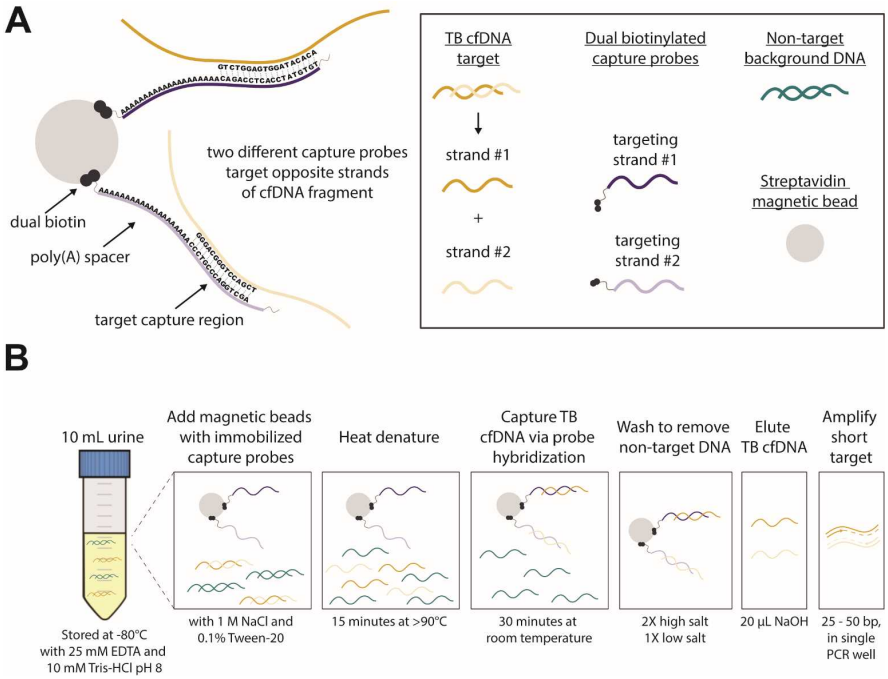

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hybridization, urine, tuberculosis, cell-free DNA, transrenal DNA, magnetic beads, sample preparation, DNA extraction, cfDNA, trDNA, DNA purification, sequence-specific purification, molecular diagnostics, magnetic capture hybridization, MCH-PCR

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35292

MATERIALS TEXT

Probe, primer, and target sequences:

Oligo	Sequence
Positive control (50 nt)	5′-CGAACCCTGCCCAGGTCGACACCATTCAACACATAGGTGAGGTCTGCTAC-3′
Reverse complement positive control (50 nt)	5′-GTAGCAGACCTCACCTATGTGTTGAATGGTGTCGACCTGGGCAGGGTTCG-3′
IS6110 TB genomic target (40 nt)	5′-CGAACCCTGCCCAGGTCGACACATAGGTGAGGTCTGCTAC-3′
Biotin probe #1 (BP1, targets positive control)	5′-/52-Bio/AAAAAAAAAAAAAAAAAAAACAGACCTCACCTATGTGT/3SpC3/-3′
Biotin probe #2 (BP2, targets reverse complement)	5′-/52-Bio/AAAAAAAAAAAAAAAAAAAACCCTGCCCAGGTCGA/3SpC3/-3′
Forward primer	5′-CGAACCCTGCCCAGGTCGA-3′
Reverse primer	5′-GTA+GCAGA+CCTCACCTATGTGT-3′

Note: All DNA sequences were ordered HPLC-purified from Integrated DNA Technologies (IDT).

Genomic target:
Our primers and probes are designed to target IS*6110*, an insertion sequence with variable copy number present in >99% of TB strains. The probes and primers are designed to target a subregion of IS*6110* shown to be conserved across and specific to the *Mycobacterium tuberculosis* complex. Design of capture probes for new targets is straightforward (see "Application to new targets" below).

Probe design:
Two different biotinylated probes are used to target both strands of the dsDNA target region. The probes are truncated versions of the primers. Each probe includes a 5′ dual biotin modification (“/52-Bio/”) for immobilization on streptavidin-coated magnetic beads, a 20 nt poly(A) spacer to reduce steric hindrance during hybridization, and a 3′ spacer (“/3SPC3/”) to block amplification of any residual probes during PCR. Dual biotinylated capture probes increase recovery compared to single biotinylated probes by reducing reliance on probe density on the bead surface (a key variable affecting efficiency of surface-based hybridization), improving thermostability of the bead-probe linkage, and eliminating interference by endogenous biotin in urine.

Primer design:
The reverse primer contains two locked nucleic acid (LNA) bases (indicated by “+X”) to precisely match the forward and reverse primer melt temperatures (T_m) to discourage non-specific amplification. We selected an annealing temperature (T_a) at or slightly above the measured primer T_m to encourage specific amplification without compromising amplification efficiency.

Positive control design:
The synthetic positive control (50 bp) was used as a spike-in control during extraction and for the PCR standard curves. It was designed to be amplifiable by the same primer set but distinguishable from the native TB target sequence (40 bp) by melt analysis so that any potential contamination with the positive control would not lead to false positives.

Application to new targets:

Design of new capture probes: Designing capture probes for new targets is straightforward. In our experience, implementation of new capture probes does not require design iterations or assay optimization and the hybridization method has high, robust recovery independent of the probe sequences. To minimize target footprint, we recommend using truncated versions of the forward and reverse PCR primers as probe sequences (see figure below). This also minimizes secondary structure, which should be limited in well-designed primers. Nucleotides should be removed from the 5′ end of both primers

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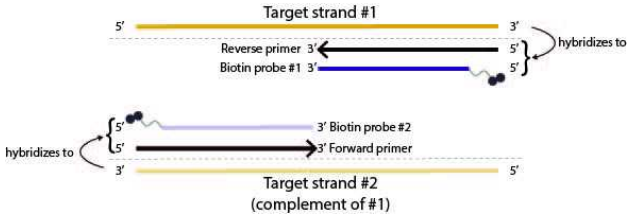
until lengths of 15 – 20 nt are reached, and the melt temperatures of the two probes are similar (as a rule of thumb, ~60°C as calculated in hybridization conditions of 1 M NaCl and 2 nM probe). In addition to the target-specific binding region, probes should contain a 5′ dual biotin modification, a 20 nt poly(A) spacer between the biotin modification and the target-specific binding region, and a 3′ carbon spacer as described above. Capture probes should be ordered with HPLC purification.



Use of existing PCR primers: In general, existing primer designs should work in combination with hybridization capture. For urine cfDNA, it is important to keep the PCR amplicon as short as possible to maximize the fraction of fragmented cfDNA that can be successfully amplified (ie, that contains both intact primer sites). For new targets, we recommend using PCR conditions with high concentration of the buffering component (eg, 80 mM Tris-SO_4 used here). Because the exact eluate volume varies slightly, we found that partial neutralization of the eluate had the least effect on downstream amplification (ie, in experiments where we controlled the eluate pH, the PCR tolerated slightly basic eluate better than slightly acidic eluate). Different primer pairs are affected uniquely by PCR conditions like pH, and may be affected by variations in eluate pH if the PCR buffer capacity is low. If this is the case and inhibtion of amplification is observed, we suggest adding more Tris-HCl to the PCR master mix.

Design of new positive controls: To easily identify contamination and prevent false positives, we recommend adding a 10 - 20 bp spacer region in between the primer binding sites on your target of interest to design a synthetic positive control that can be distinguished from the native target of interest by post-amplification melt curve analysis.

Required materials:

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Material	Supplier	Catalog #
0.5 M EDTA pH 8	Quality Biological	351-027-721EA
1 M Tris-HCl pH 8	Thermo Fisher	J22638
Fisherbrand 10-SG Urine Reagent Strips (optional)	Thermo Fisher	23-111-262
Dynabeads MyOne Streptavidin C1	Invitrogen (Thermo Fisher)	65001
Capture probes	IDT	See table above
Molecular biology grade water	Corning	46-000-CM
5 M NaCl	Quality Biological	351-036-101
10% Tween-20	Teknova	T0710
100 mM NaOH	VWR	BDH7219-1
100 mM HCl	VWR	BDH7200-1
OneTaq Hot Start DNA Polymerase	NEB	M0481
OneTaq GC Reaction Buffer Pack	NEB	Included with OneTaq
dNTPs	NEB	N0447
EvaGreen	Biotium	31000
Low-EDTA TE (TLE) pH 8	Quality Biological	351-324-721
Capture probes	IDT	See table above
Forward primer	IDT	See table above
Reverse primer	IDT	See table above
DNA LoBind tubes (1.5 mL and 15 mL)	Eppendorf	022431021 and 0030122208
Sterile urine collection containers	any	n/a
10 mL serological pipets	any	n/a
Sterile filtered pipette tips	any	n/a
0.2 mL 8-Tube PCR Strips without Caps, low profile, clear	Bio-Rad	TLS0801
0.2 mL Flat PCR Tube 8-Cap Strips, optical, ultraclear	Bio-Rad	TCS0803

Note: It is likely okay to substitute common reagents (with the exception of magnetic beads, PCR reagents, and consumables) with those from other suppliers. In general, we recommend using molecular biology-grade, nuclease-free reagents.

Required equipment:

- -80°C freezer (if storing urine samples prior to analysis)
- P20, P100, and P1000 pipettes
- Pipette controller for serological pipettes
- Centrifuge with 15 mL conical tube rotor, capable of at least 8000g
- Dry bath with 15 mL tube block (or water bath)
- Tube rotisserie with 1.5 mL and 15 mL tube adapters
- Mini centrifuge
- Vortex mixer
- 1.5 mL magnetic tube rack (we use Invitrogen DynaMag-2, catalog #12321D)
- Real-time PCR thermocycler (we use Bio-Rad CFX96 TouchReal-Time PCR Detection System)

BEFORE STARTING

Prepare ahead of time:

1. High salt wash buffer (1M NaCl, 10 mM Tris-HCl pH 8, 0.05% Tween-20)

To make 500 mL:

- 392.5 mL molecular biology grade water
- 100 mL 5M NaCl
- 5 mL 1M Tris-HCl pH 8
- 2.5 mL 10% Tween-20
- Aliquot and store at room temperature

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2. Low salt wash buffer (15 mM NaCl, 10 mM Tris-HCl pH 8, no Tween-20)

To make 500 mL:
- 493.5 mL molecular biology grade water
- 1.5 mL 5M NaCl
- 5 mL 1M Tris-HCl pH 8
- Aliquot and store at room temperature

3. Pre-mixed capture probes (50 µM BP1 and 50 µM BP2 in TLE)

To make 100 µL:
- Resuspend dry capture probes BP1 and BP2 at 100 µM in TLE pH 8
- Combine 50 µL BP1 and 50 µL BP2 and mix well
- Aliquot and store at -20°C

4. 1X TLE with 0.5% Tween (if storing beads after probe immobilization)

To make 1 mL:
- 50 µL 10% Tween-20
- 950 µL 1X TLE
- Store at 4°C

Important note: Controls and precautions to avoid false positives

Every experiment should include a positive control (eg, pooled TB-negative urine spiked with 10^3 copies of dsDNA synthetic positive control template) and negative control (eg, water without spiked target) that are run throughout the entire extraction process alongside clinical urine samples.

To avoid false positives due to contamination, the synthetic positive control (50 bp, used as a spike-in control during extraction and for PCR standard curves) is designed to be amplifiable by the same primer set but distinguishable from the native TB target sequence (40 bp) by melt analysis. Any contamination with the positive control will be easily identifiable and will not result in false positives. In addition, we maintain good laboratory practices to limit contamination (eg, separating pre- and post-PCR rooms, regular decontamination of work surfaces and pipettes, sterile filtered pipette tips, aliquoting reagents into single-use volumes).

To avoid false positives due to nonspecific amplification, we precisely matched the T_m of the primers using LNA substitutions and selected a PCR annealing temperature at or slightly above the measured primer T_m to encourage specific amplification without compromising amplification efficiency. Most non-target DNA should be removed by sequence-specific hybridization capture, but even very small amounts of residual non-target DNA (due to non-specific adsorption to bead surface, which we were unable to completely eliminate by blocking with BSA, sheared salmon sperm DNA, or Denhardt's solution) has the potential to lead to non-specific amplification when detecting very low concentrations of target cfDNA. The given primers and PCR conditions typically have no nonspecific amplification up to at least 45 PCR cycles.

Urine collection and storage

1 Before urine collection, prepare 15 mL Eppendorf DNA LoBind tubes with 500 µL 0.5 M EDTA and 100 µL 1M Tris-HCl pH 8.

2 Collect urine sample in a sterile container.

3 Immediately after urine collection, add 10 mL of urine to each prepared 15 mL tube. The final concentration of EDTA and Tris-HCl will be ~25 mM and ~10 mM, respectively.

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CRITICAL STEP: *EDTA protects cfDNA from degradation by inactivating DNAses in urine. Tris-HCl buffers urine pH for storage. To minimize the risk of cfDNA degradation, it is important to add EDTA and Tris-HCl as soon as possible after urine collection.*

4 Mix by inversion.

5 Freeze at -80°C until analysis.

6 Immediately before analysis, thaw urine at 37°C.

7 Mix urine gently by inversion.

8 Test with Fisherbrand 10-SG Urine Reagent Strips (optional).

9 Centrifuge urine for 5 minutes at 8000g to pellet cell debris.

00:05:00

10 Transfer cell-free urine supernatant to new 15 mL Eppendorf DNA LoBind tubes.

Alternative: *If possible at collection site, can centrifuge and transfer cell-free urine to new tubes prior to freezing.*

Immobilize capture probes on magentic beads

11 Vortex Dynabeads MyOne Streptavidin C1 for 30 seconds to ensure that beads are evenly dispersed in solution.

12 Pipette beads into a 1.5 mL Eppendorf DNA LoBind tube. Prepare 50 µL (0.5 mg) beads per 10 mL urine sample to be analyzed (eg, for 16 samples, prepare 800 µL beads).

13 Wash beads three times with an equal volume of high salt wash buffer (1M NaCl, 10 mM Tris-HCl pH 8, 0.5% Tween-20).

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13.1 Place beads on magnetic rack for 1 minute.

00:01:00

13.2 Remove and discard supernatant by pipette.

13.3 Add equal volume of high salt wash buffer (1M NaCl, 10 mM Tris-HCl pH 8, 0.05% Tween-20).

13.4 Vortex for 5 seconds.

13.5 Spin down briefly.

13.6 Repeat twice for a total of three washes. go to step #13.1

14 Resuspend in equal volume of high salt wash buffer (1M NaCl, 10 mM Tris-HCl pH 8, 0.05% Tween-20).

15 Pre-mix dual biotinylated capture probes BP1 and BP2 in TLE to a final concentration of 50 µM BP1 and 50 µM BP2.

Note: *We recommend pre-mixing probes in bulk ahead of time, aliquoting, and freezing at -20°C until use.*

16 Add pre-mixed biotinylated capture probes BP1 and BP2 to side wall of bead tube, close lid, and immediately vortex for 5 seconds. Use 0.5 µL of pre-mixed 50 µM probes (25 pmol of each probe) per 50 µL beads (eg, use 8 µL probe mix for 16 samples).

CRITICAL STEP: *It is important to pre-mix the probes and vortex the beads immediately to ensure homogenous probe distribution on the beads. If probes are added sequentially, binding to the beads and subsequent recovery will favor the probe that is added first.*

17 Rotate for 15 minutes at room temperature to immobilize probes on beads.

00:15:00

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18 Spin down briefly.

19 Wash beads three times with an equal volume of high salt wash buffer (1M NaCl, 10 mM Tris-HCl pH 8, 0.5% Tween-20).

19.1 Place beads on magnetic rack for 1 minute.

00:01:00

19.2 Remove and discard supernatant by pipette.

19.3 Add equal volume of high salt wash buffer (1M NaCl, 10 mM Tris-HCl pH 8, 0.05% Tween-20).

19.4 Vortex for 5 seconds.

19.5 Spin down briefly.

19.6 Repeat twice for a total of three washes. go to step #19.1

20 Resuspend beads in an equal volume high salt wash buffer (1M NaCl, 10 mM Tris-HCl pH 8, 0.05% Tween-20).

Alternative: *Beads can be prepared ahead of time and stored at 4°C for 1 - 2 weeks. If storing for future use, resuspend beads in an equal volume 1X TLE with 0.5% Tween-20 instead of high salt wash buffer and store at 4°C.*

Capture TB urine cfDNA by hybridization

21 Add 2.5 mL 5 M NaCl (final concentration 1 M), 127 µL 10% Tween-20 (final concentration 0.1%) and 50 µL prepared beads to each 10 mL sample. Vortex beads well to ensure that they are evenly resuspended before adding to urine.

22 If spiking in positive control, add it now.

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23 Mix well by inversion.

24 Denature for 15 minutes in dry bath with 15 mL tube block preheated to 120°C (urine temperature should reach >90°C).

00:15:00

Alternative: *Denature in 90°C water bath.*

25 Rotate for 30 minutes at room temperature to hybridize TB-specific cfDNA to capture probes.

00:30:00

Wash to remove urine inhibitors and non-target DNA

26 Centrifuge for 5 minutes at 5000g to pellet beads.

00:05:00

27 Remove and discard all but approximately 1 mL supernatant using 10 mL serological pipette.

28 Resuspend beads in remaining supernatant using P1000 pipette and transfer to 1.5 mL Eppendorf DNA LoBind tube.

29 Place on magnetic rack for 1 minute.

00:01:00

30 Remove and discard supernatant by pipette.

Note: *Some urine samples (often those with blood or abnormally high protein concentrations) may cause beads to aggregate poorly on the magnet. If this is the case, we suggest leaving the sample on the magnet for longer (5 min) and/or using a minicentrifuge to help pellet the beads before removing the supernatant here and in subsequent wash steps. It is okay to leave more residual volume to avoid bead loss.*

This problem occurs infrequently, but we have still successfully extracted cfDNA from samples despite increased residual volumes and bead loss during the wash steps.

31 Remove tube from magnetic rack.

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32 Add 1 mL high salt wash buffer (1 M NaCl, 10 mM Tris-HCl pH 8, 0.05% Tween-20) and wash by inverting 10-20 times, or until no bead aggregate is left on tube wall. Do not vortex.

33 Spin down briefly.

34 Place on magnetic rack for 1 minute.

00:01:00

35 Remove and discard supernatant by pipette.

36 Remove tube from magnetic rack.

37 Add 1 mL high salt wash buffer (1 M NaCl, 10 mM Tris-HCl pH 8, 0.05% Tween-20) and wash by inverting 10-20 times, or until no bead aggregate is left on tube wall. Do not vortex.

38 Spin down briefly.

39 Place on magnetic rack for 1 minute.

00:01:00

40 Remove and discard supernatant by pipette.

41 Remove tube from magnetic rack.

42 Add 1 mL low salt wash buffer (15 mM NaCl, 10 mM Tris-HCl pH 8, no Tween-20) and wash by inverting 10-20 times, or until no bead aggregate is left on tube wall. Do not vortex.

43 Spin down briefly.

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44 Place on magnetic rack for 1 minute.

00:01:00

45 Remove and discard supernatant by pipette.

46 Spin down again.

47 Place on magnetic rack and remove as much liquid as possible using P20 pipette.

CRITICAL STEP: *Because of the small elution volume in the next step, it is important to remove as much of the residual wash buffer as possible, while avoiding bead loss.*

Elute purified TB cfDNA

48 Prepare fresh 20 mM NaOH by adding 400 µL water to 100 µL 100 mM NaOH.

49 Add 20 µL freshly-prepared 20 mM NaOH to beads to elute purified TB-specific cfDNA.

50 Vortex for 5 seconds.

51 Spin down briefly.

52 Place on magnetic rack.

53 Transfer as much supernatant as possible (usually 20 – 21 µL; set P20 pipette to maximum volume) directly to PCR well or to new Eppendorf DNA LoBind tube. This contains purified TB-specific cfDNA.

CRITICAL STEP: *To maximize recovery, make sure to transfer as much eluate as possible to PCR. Avoid transferring any beads to PCR because they can lead to PCR inhibition.*

Note: *Some urine samples (often those with blood or abnormally high protein concentrations) may also make it difficult to avoid bead transfer to PCR. If this is the case, we suggest leaving the sample on the magnet for longer (5 min) and/or using a minicentrifuge to help pellet the beads before eluting. It is okay if the eluted volume is <20 µL or if a small amount of beads are transferred to PCR (although it will lead to PCR inhibition).*

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This problem occurs infrequently, but we have still successfully detected cfDNA in samples despite reduced elution volumes and PCR inhibition caused by minor transfer of beads to PCR.

54 Partially neutralize with 3.5 µL 100 mM HCl.

Note: *Since the exact elution volume varies across samples, the PCR buffer will adjust to final pH, and tolerates sligtly basic pH better than acidic pH. For this reason, we also recommend using PCR conditions with a high buffering capacity (eg, 80 mM Tris-SO4 used here).*

55 Proceed directly to PCR.

Alternative: *We recommend proceeding directly to PCR, but if you cannot amplify immediately, add 2 µL 10X TLE and store at 4°C.*

Quantify by PCR

56 Prepare a PCR master mix according to the table below. Each reaction should contain 1.25 U OneTaq Hot Start DNA Polymerase (NEB), 1X NEB OneTaq GC Reaction Buffer (80 mM Tris-SO_4, 20 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 5% glycerol, 5% DMSO, 0.06% IGEPAL CA-630, 0.05% Tween-20, pH 9.2), 0.8 mM dNTPs (NEB), 0.4X EvaGreen (Biotium), 200 nM forward primer, and 200 nM reverse primer. The total reaction volume should be 50 µL, 26 µL of which is master mix.

	concentration			volume			
Component	**stock**	**final**	**units**	**per rxn**	**units**	**master mix**	**units**
OneTaq GC Reaction Buffer	5	1	X	10	uL		uL
dNTPs	40	0.8	mM	1	uL		uL
Forward primer	100	0.2	uM	0.1	uL		uL
Reverse primer	100	0.2	uM	0.1	uL		uL
EvaGreen	20	0.4	X	1	uL		uL
OneTaq HotStart Polymerase	5	1.25	U	0.25	uL		uL
Water				13.55	uL		uL
Total master mix volume				26	uL		uL
Hybridization output				~24	uL	n/a	
Total reaction volume				50	uL	n/a	

57 For the hybridization output, add 26 µL master mix to the entire neutralized output (~24 µL) of each experimental sample.

58 For the standard curve (0, 10, 10^2, 10^3, 10^4, and 10^5 copies positive control, in triplicate), add 19 µL nuclease-free water and 26 µL master mix to 5 µL DNA standard.

59 Amplify in CFX96 Touch Real-Time PCR Detection System (Bio-Rad) with an initial incubation of 94°C for 3 min followed by 45 amplification cycles (94°C for 30s, 64°C for 30s, and 68°C for 1 min).

60 Measure amplicon T_m by post-amplification melt curve analysis from 65°C to 95°C in 0.5°C increments every 5 seconds.

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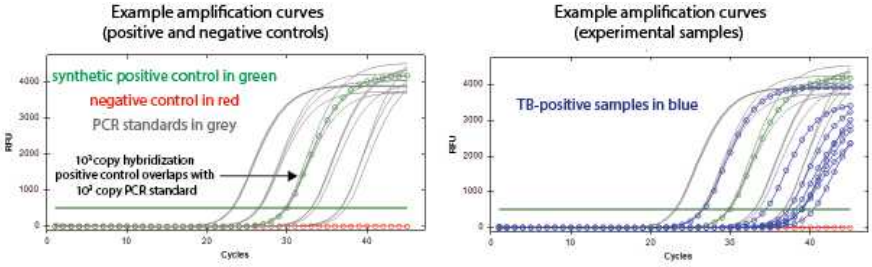
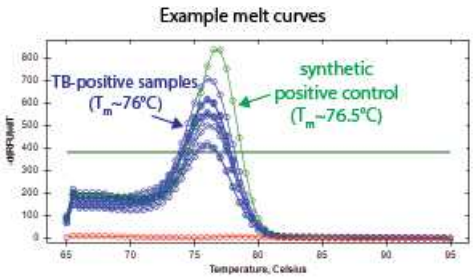
61 Determine C_q values at a threshold of 500 RFU and calculate recovered copies using the standard curve.

- Recovery of the positive control should be **near 100% (80 - 120%)**. Calculated percent recovery may occasionally be >100% due to inherent variability of PCR; a small shift in C_q (positive control or PCR standards) leads to an exponential change in calculated percent recovery. The positive control PCR amplification curve should be similar to the corresponding PCR standard.
- There should be no amplification of the negative control or PCR NTCs up to at least 45 cycles.
- Detected copy number in experimental samples will depend on the target of interest and may vary by several orders of magnitude across biological replicates. In preliminary clinical testing with our IS6110 TB target, we observed copy numbers ranging from <1 - 3E4 in 10 mL urine (median 147 copies).



62 Confirm that amplicon T_m for clinical samples matches that of expected target (should be 0.5°C below the positive control T_m).

- The T_m of TB-positive samples should be ~0.5°C below the T_m of the positive control.
- If any samples amplify with incorrect T_m, they should be considered TB-negative.



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