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Lyophilized bead multiplex assays for CT, NG, and TV detection

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Background

Chlamydia trachomatis (CT), *Neisseria gonorrhoeae* (NG), and *Trichomonas vaginalis* (TV) cause the three most prevalent sexually transmitted infectious diseases in developed countries, contributing to approximately 300 million new infections worldwide each year. CT and NG infections are the leading cause of pelvic inflammatory disease and infertility worldwide; TV is linked to higher rates of HIV transmission, low birth weight, and infertility. Due to the challenges of culturing and detecting these pathogens with immunoassays, DNA-based molecular tests have become instrumental for pathogen detection, and current trends in molecular testing for CT, NG, and TV have moved toward point-of-care tests.

Lyophilization provides benefits required for point-of-care tests, such as ambient storage, extended shelf life, simplified downstream workflow, and variable formats. A lyophilized-bead format enables an assay to be placed in sealed consumables and stored at room temperature for up to two years.

An additional consideration is the need for endogenous and exogenous controls. Exogenous in-process controls can validate the performance of workflows culminating in a qPCR reaction and provide flexibility to test developers. An ideal exogenous in-process control is nonhomologous to human or human pathogen genome sequences and does not align with primers or probes designed for pathogen detection.

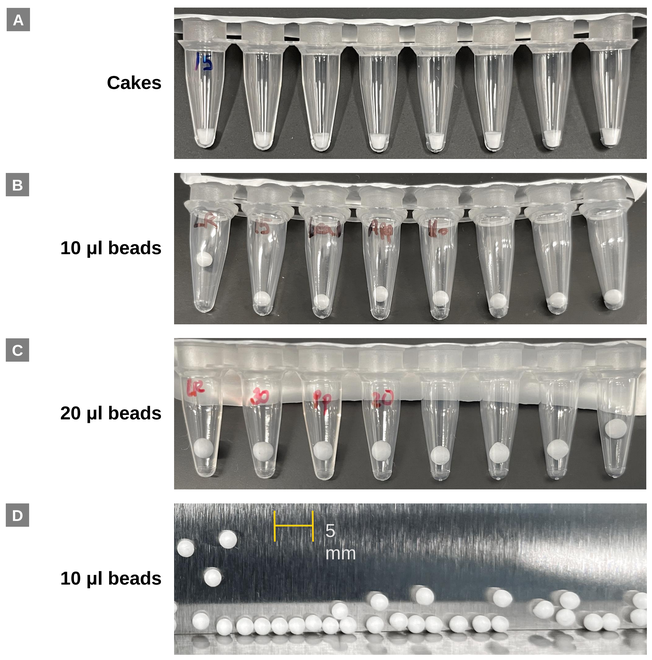
This study aims to develop a lyophilized-bead-format assay with an ideal exogenous control and establish its validity as an option for the accurate detection of CT, NG, and TV.

Materials

Lyo-Ready PrimePath™ Probe qPCR Mix, GPR was designed to be directly incorporated into a lyophilization process, with a *Taq* hot-start polymerase premixed with an excipient so that no additional excipients are required. Assay primers and probes can be incorporated into the solution prior to dispensing.

A universal process control was developed by designing a unique 1 kb (kilobase) exogenous sequence from a shortest common super-sequence (SCS) of 14-mers. The exogenous sequence was extensively tested in silico not to match any of the genomes represented so far in the databases of human pathogens (including bacteria, viruses, fungi, and protozoa) or nonpathogens (including genomes for humans, mice, and other common hosts).

Assays were designed for each of five targets: CT, NG, TV, RNase P (RP), and the exogenous control (Exo). A synthetic DNA control containing targets for CT, NG, TV, RP, and Exo was used to establish an analytical limit of detection (LOD) by qPCR. The 5-plex assay, lyophilized in beads, was hydrated and tested for linearity over an 8-log (1–1 x 10^7) range of the synthetic DNA control. Further, the lyophilized bead + qPCR assay was tested with a proficiency sample by extracting DNA from ZeptoMetrix NATtrol CT/NG/TV Positive Control and spiking in the exogenous DNA control. qPCR was performed on the extracted DNA and human cDNA.



Lyophilized PrimePath Probe qPCR cakes and beads were produced by lyophilization of Lyo-Ready PrimePath Probe qPCR solution. Panel A. Cakes were made by dispensing solution into the consumable of choice followed by lyophilization. **Panels B and C.** Beads were formed by dispensing droplets of a desired volume of solution into liquid nitrogen for a fast freeze in a spherical shape followed by lyophilization. **Panel D.** 10 µl beads on a tray with a 5 mm ruler in yellow for size reference. The 10 µl beads measure 2.47 mm ± 0.12 mm in size. The 20 µl beads, containing primers and probes, measure 3.18 mm ± 0.17 mm in size.

Results

1 Analytical LODs as low as 5 copies/rxn

Positivity	10 cp/rxn	5 cp/rxn	1 cp/rxn	0.5 cp/rxn
CT	20/20	20/20	18/20	14/20
NG	20/20	20/20	18/20	15/20
TV	20/20	20/20	18/20	15/20
RP	20/20	20/20	14/20	13/20
Exo	20/20	20/20	18/20	15/20

Figure 1. Analytical LOD of CT, NG, TV, RP, and Exo. An analytical LOD was determined by performing 20 multiplex qPCR reactions at four different concentrations using Lyo-Ready PrimePath Probe qPCR Mix, GPR and a gene fragment template DNA containing targets for CT, NG, TV, RP, and Exo (GenScript), quantified by dPCR, on a QuantStudio 5 instrument. Primer and probe concentrations for CT, NG, TV, and Exo were 500 nM and 200 nM, respectively; primer and probe concentrations for RP were 900 nM and 300 nM, respectively. The analytical LOD for the control DNA was determined to be 5 copies/reaction (cp/rxn).

2 Reproducible amplification across assay formats

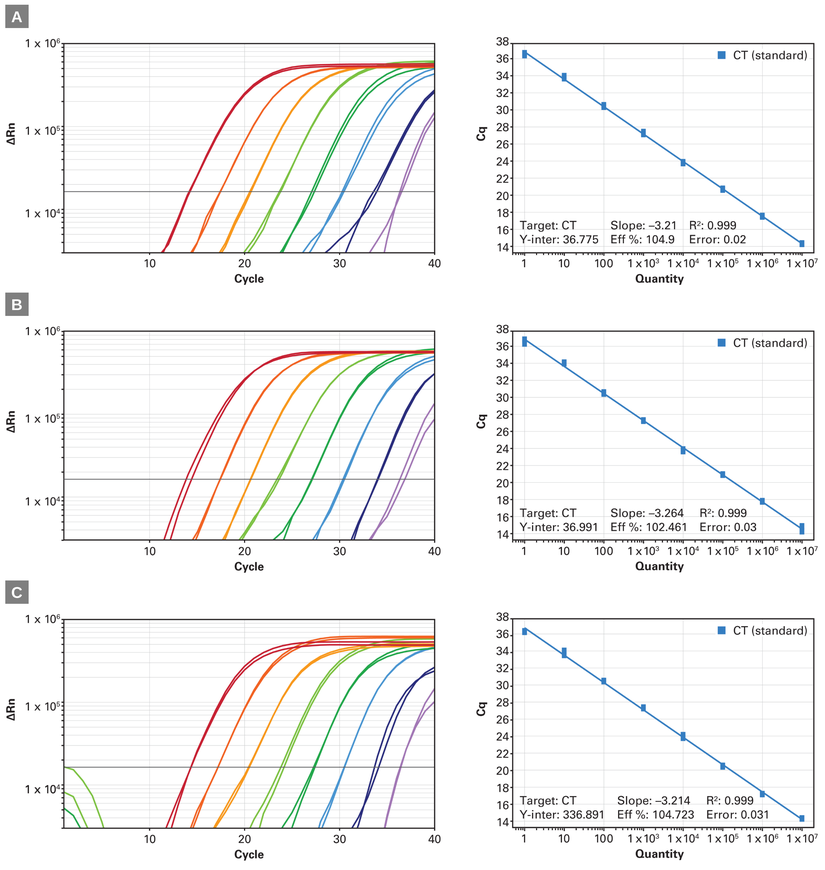


Figure 2. Linear dynamic range of Lyo-Ready PrimePath Probe qPCR Mix, GPR and Lyophilized PrimePath Probe qPCR Mix. Two replicates of an eightfold dilution series of a gene fragment template DNA were tested using primers and probes targeting CT. All panels show amplification and standard curve plots with R^2 values and PCR efficiency (Eff %). **Panel A.** Lyo-Ready PrimePath Probe qPCR Mix, GPR. **Panel B.** Lyophilized PrimePath Probe qPCR Mix (cakes). **Panel C.** Lyophilized PrimePath Probe qPCR Mix (beads containing primers and probes). Lyo-ready and Lyophilized PrimePath Probe mixes demonstrate the same consistent amplification across all dilution series points regardless of shape or freezing method.

Conclusions

- We have developed a multiplex qPCR assay in lyophilized-bead format that can identify infectious agents like CT, NG, and TV in a single reaction.
- We have included RP as a human host control, plus an exogenous in-process control, for a 5-plex target total in separate fluorescent channels.
- Linearity was observed by qPCR over an 8-log range of the synthetic DNA control for all five targets in multiplex, with good efficiencies and R^2 values
- We have shown that our lyophilized beads with assay produce a positive qPCR result when combined with a proficiency sample.

3 5-plex assay linearity

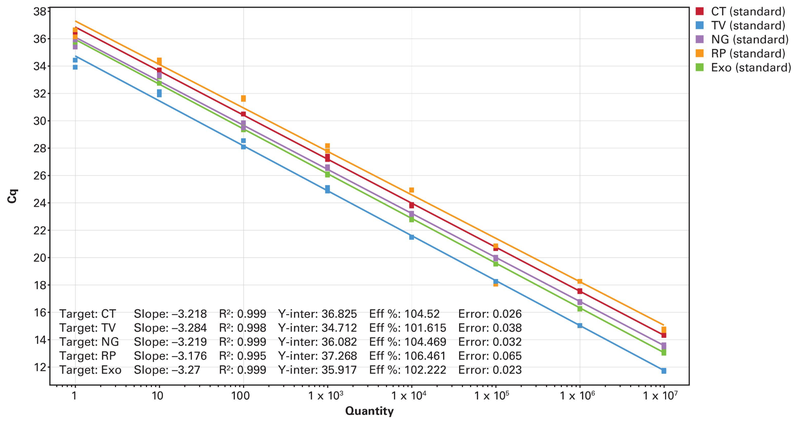


Figure 3. Performance of Lyo-Ready PrimePath Probe qPCR Mix, GPR in a 5-plex assay. Linearity was observed by qPCR over an 8-log range of the synthetic DNA control for all five targets in multiplex with good efficiencies and R^2 values. Lyo-Ready PrimePath Probe qPCR Mix, GPR was combined with primer and probe sets for CT, NG, TV, RP, and Exo. Lyo-Ready PrimePath Probe qPCR Mix, GPR maintained comparable performance for all five genes in the multiplex assay, as demonstrated by the standard curve plots, R^2 values, and qPCR efficiencies for each individual gene.

4 Unbiased 5-plex assay amplification

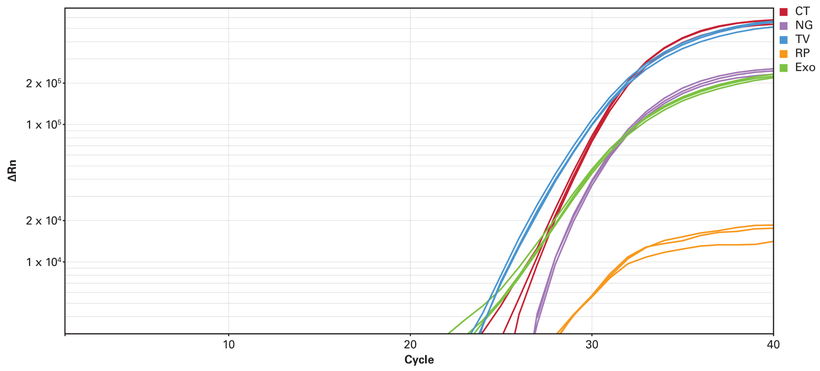


Figure 4. Five target synthetic DNA detection using beads with assays premixed. Lyophilized PrimePath Probe qPCR Mix beads containing primers and probes were used to detect DNA from a 5-plex reaction following amplification of a synthetic DNA control containing targets CT, NG, TV, RP, and Exo at 1,000 copies/reaction.

5 Successful detection of CT, NG, and TV from a proficiency sample

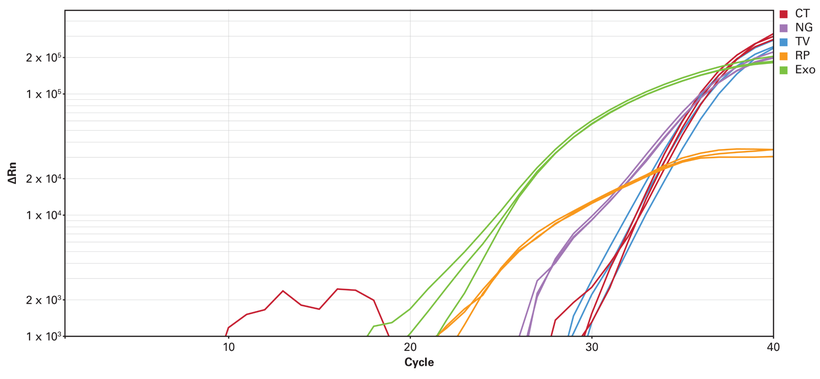


Figure 5. Detection CT/NG/ and TV from a molecular diagnostics (MDx) proficiency sample. Lyophilized PrimePath Probe qPCR Mix beads containing primers and probes were used to detect DNA from a proficiency sample. DNA was extracted from ZeptoMetrix CT/NG/TV Positive Control using NucleoMag Pathogen. Exogenous extraction control was spiked in at 100,000 copies after the lysis step. Human cDNA was spiked in at 100 ng per reaction after extraction. All targets were amplified and detected by qPCR using DNA extracted from ZeptoMetrix NATtrol CT/NG/TV Positive Control with spiked-in Exo. The detection of the exogenous sequence in the qPCR multiplex reaction indicated that the extraction was successful.

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