FY23 Technical Report NDSC (Final)

Project Title: **A tool for cheap and rapid tracking of soybean inoculant populations in field soil**

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Objective 1: Evaluate improvement of sensitivity with TaqMan probes and finalize technology platform

In the previous year, we set out to explore dPCR or qPCR as technology platforms for an assay to enumerate soybean rhizobia (symbiotic *Bradyrhizobium japonicum*) in farmer's soil. Work from FY22 culminated in a successful qPCR assay with a sensitivity limit of ~1000 rhizobia per gram (See FY22 report, Figure 1) (Assay Version 1.0). Development of an assay with a new technology platform, digital PCR (dPCR) which was advertised to have enhances specificity and sensitivity to qPCR, was also attempted. However, dPCR assays were unsuccessful due to high amounts of nonspecific signal in negative controls. Conversations with the manufacturer (QIAgen) indicated that incorporating TaqMan probes into the assay would overcome this issue in dPCR. TaqMan probes are a modification to amplicon-based molecular detection methods (qPCR/dPCR) that provide an added layer of specificity by binding to the amplicons and creating a detectable fluorescent signal when bound. Since TaqMan probes can also be utilized in qPCR and have the potential to increase sensitivity of our assay, we sought to evaluate incorporation of TaqMan probe technology into both qPCR and dPCR assays (Assay Version 2.0). Therefore, our first Objective of FY23 was to investigate the incorporation of TaqMan probes into qPCR and dPCR assays to both 1) finalize the selection of technology platform, and 2) evaluate improved sensitivity with their use.

TaqMan probe evaluation

Two approaches to TaqMan probe design were explored.:

1) A custom TaqMan probe assay was designed by ThermoFisher Scientific and tested. This assay however was ineffective and showed significant amplification in non-rhizobia control samples indicating a lack of specificity (data not shown).

2) We manually designed a TaqMan probe to incorporate with our previously successful primer sets utilized in the Version 1.0 qPCR assay (Figure 1).

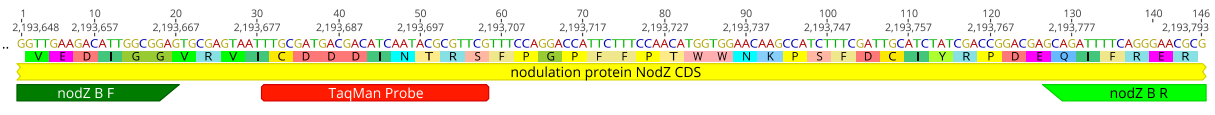

Figure 1. Design of TaqMan probe for incorporation into qPCR assay. Primer sequences for qPCR assay in green and the sequence of the TaqMan probe is in red.

To evaluate the accuracy of the new 2.0 TaqMan probe assay, we tested it using samples that were previously used to evaluate and calibrate the 1.0 qPCR assay. Overall, the 2.0 assay proved reliable and showed highly similar results to the qPCR assay with the same samples (Figure 2).

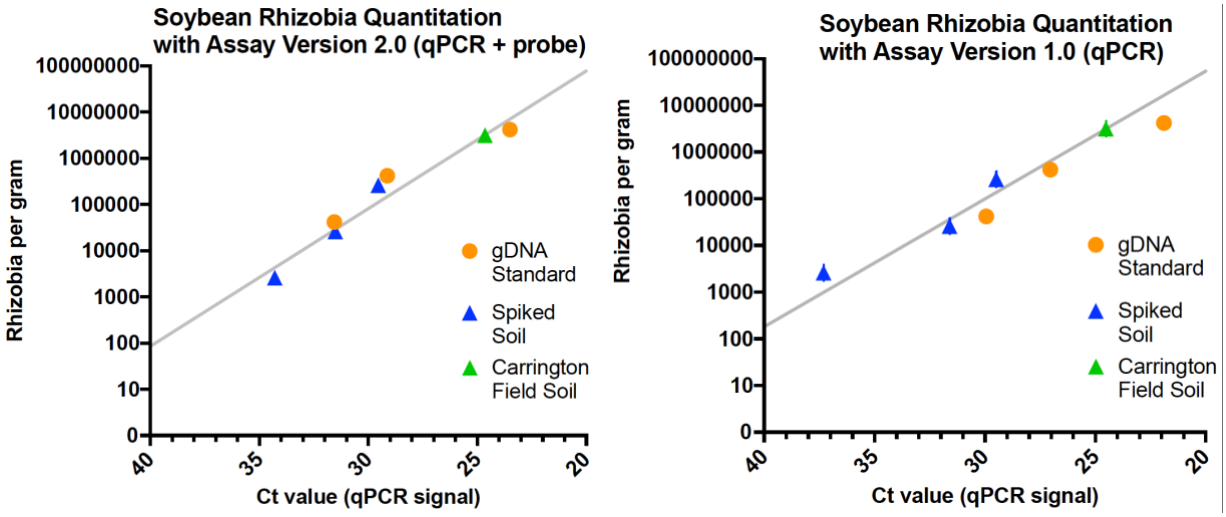

Figure 2. Assay Version 2.0 results using qPCR platform (left), Version 1.0 results (right)

Next, we evaluated the new 2.0 TaqMan probe assay with dPCR using the same samples. While the assay was now successful using dPCR when the probes were incorporated, the sensitivity of the assay was vastly lower than qPCR (Figure 3). These data, combined with the higher reagent cost of dPCR lead us to finalize qPCR as the best platform for our assay.

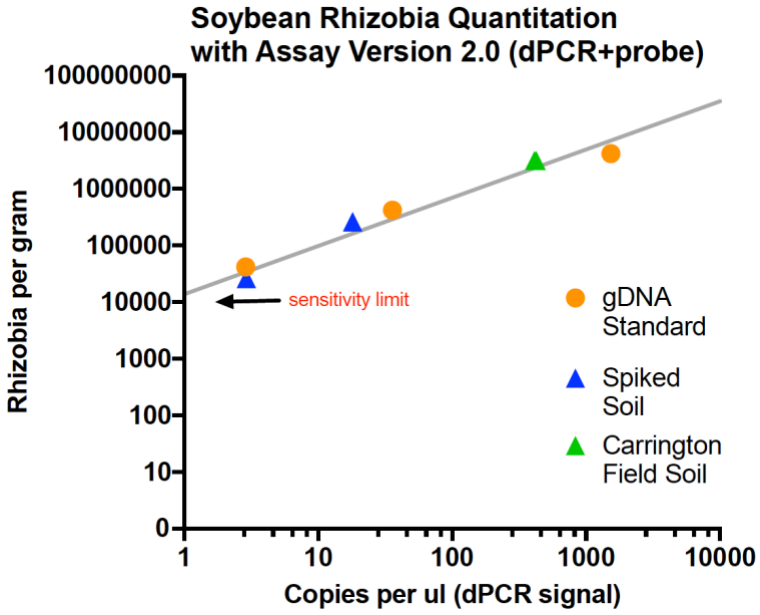

Figure 3. Assay Version 2.0 results using dPCR platform

Finally, we explored the capacity for increased sensitivity in qPCR using the new 2.0 TaqMan probe assay by using undiluted DNA samples from soil extractions. Previously, undiluted samples had too much background noise using the 1.0 assay and as a result samples needed to be diluted from ~100 ng/uL from the soil extraction to 10 ng/uL before the qPCR assay. Using the 2.0 TaqMan probe assay, undiluted samples were successfully detected without background noise and led to an enhanced sensitivity of the assay from ~1000 rhizobia per gram to ~100 rhizobia per gram.

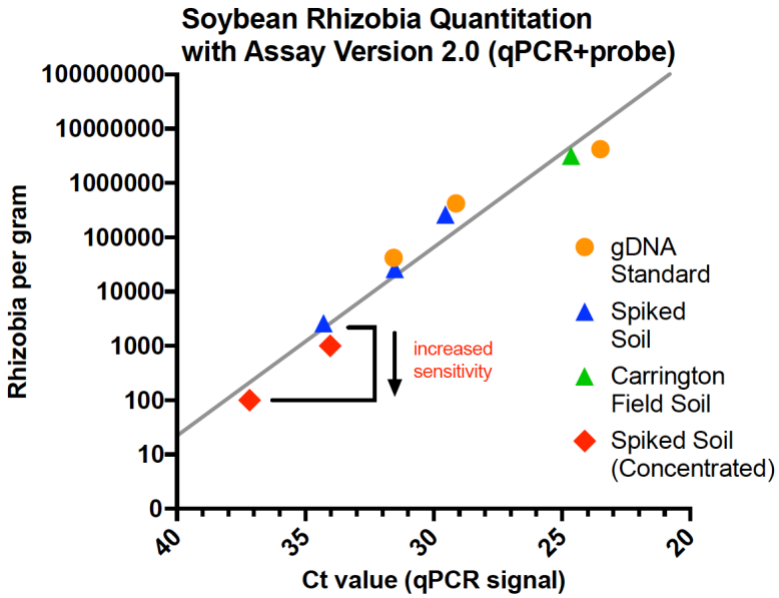

Figure 4. Assay Version 2.0 results using qPCR platform with concentrated (undiluted) DNA samples

Objective 2: Establish reliability using different soil types and sampling procedures, and optimize as necessary

We had two goals with evaluating the reliability of the NDSoy2.0 assay:1) evaluating the reliability of the assay with different soil types from the state, and 2) establishing the required sampling procedures to ensure the reliability of the assay.

Assay reliability across soil-types

To assess the reliability of the assay using different soils, we utilized previously assayed rhizobium-free soil from Central Grasslands REC (Central ND) and new soil from Williston REC (Western ND) that was expected to have limited rhizobium populations based on agronomic history in a rhizobium spiked soil experiment. Rhizobia were spiked into each of the soils in a 10-fold dilution series, DNA was extracted from each of the soils, and the soils were assayed with the NDSoy2.0 assay to estimate the quantity of rhizobia in each soil. The results from the experiment are shown in Figure 5A and 5B. While the central grasslands showed the expected linear quantification across the dilution, the soils from Williston deviated from a linear increase in quantification (Ct) as rhizobia concentration increased. This resulted in a significant

underestimation of the rhizobia in Williston soil samples relative to Central Grasslands at high levels of rhizobia, but a similar quantification when low levels of rhizobia were present). Combined, the non-linear amplification across the assay as well as the reduced detection of rhizobia in Williston soil indicate that the starting soil may be an important factor to consider for bias when performing the assay, and for maximum accuracy across soiltyps. Sandy soils such as those found in Williston are recognized as creating DNA extraction challenges. Future efforts should be made to normalize the quantification to the starting soiltype either through optimizing the DNA extraction procedure or introducing an approach for normalization between soiltypes.

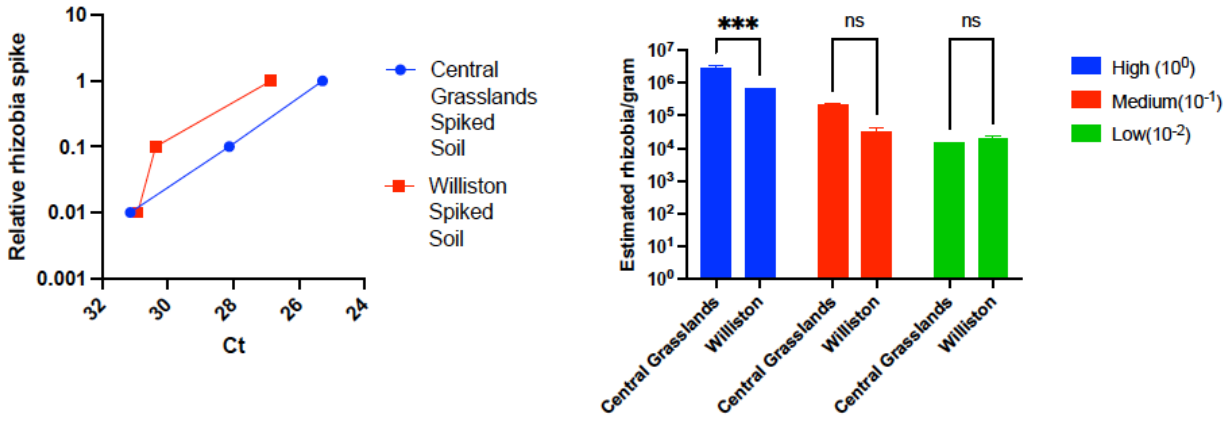

Figure 5. Comparison of efficiency of *Bradyrhizobium* detection in spiked Central Grasslands and Williston soil. A. (left) Amplification bias across log(10) dilutions of spiked rhizobia. The relative rhizobia concentration is plotted on the Y axis and the amplification cycle by NDSoy1.0 on the X axis. Accurate quantitation across dilutions is expected to produce a linear line. B. (right) estimated rhizobia present at three different levels of spiked rhizobia in rhizobia-free Central Grasslands and Williston soils. Statistical analysis was performed with a 2-way ANOVA

Robustness of Assay to Sampling Approaches

An assay to quantify rhizobia from farmers fields would be more useful if it were robust to sample handling similar to those that are used for soil chemical analysis. These differ substantially from sampling procedures routinely used for molecular analysis, in that they are often non-temperature controlled, may be shipped over several weeks and are dried prior to shipping. Whereas, routine protocol for molecular DNA analysis involves shipping on ice over 24 hours and immediately freezing before DNA extraction is performed. To evaluate the robustness of the assay to less rigorous sampling approaches that may be used for soil chemical analysis, we compared three field soils from previous soybean fields from Hettinger, Williston and Carrington RECs. The soils were shipped on ice from the RECs to NDSU following sampling, and either immediately frozen, or dried at room temperature for two weeks prior to extracting DNA and comparing rhizobia quantification. Overall, we did not observe significant differences between the two sample processing procedures, indicating that the assay is robust to different approaches to sample handling prior for soil chemical analysis, including drying the soil and leaving it at room temperature for several weeks.

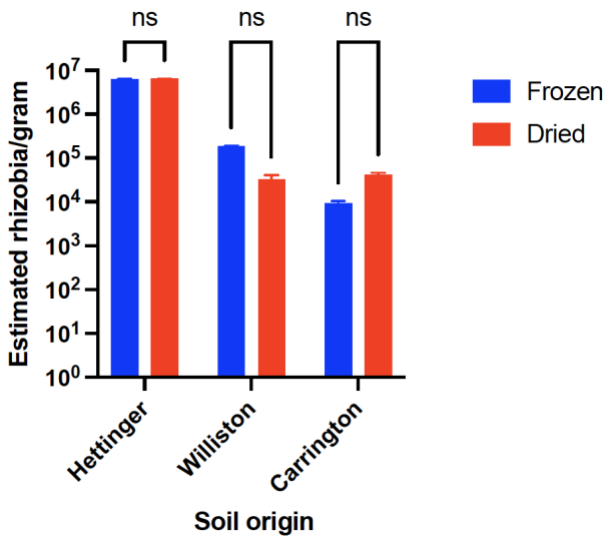

Figure 6. Comparison of sampling method and NDSoy2.0 results. Frozen indicates samples shipped overnight with cooling and immediately frozen until DNA extraction. Dried indicates samples dried and left at room temperature for 2 weeks prior to DNA extraction.

Objective 3: Test finalized assay using farmer's field soil, with a focus on inoculant survival in acidic soils from Western ND.

As a final objective of the FY23 grant, we aimed to continue testing of Farmer's field soils to gain a more robust understanding of rhizobia populations across North Dakota. FY22 data indicated significantly lower populations of rhizobia in field soils in Western ND. We considered this may lead to requiring different inoculant recommendations than those established primarily based on studied done in Eastern ND. Therefore, further data collection from fields across the state was carried out to further investigate this phenomenon. The data showed a variety of rhizobium population levels that varied from not detectable to very high populations (Figure 7). There was little difference between Western and Eastern ND when 2022 and 2023 data was taken together and rhizobium population level was compared to years since the previous soybean crop (Figure 8A). Overall, the data indicate congruence with current recommendations that rhizobium populations remain high in fields until ~the fifth year since soybean planting (4 years since last soybean crop at time of measurement) (Figure 8B) and our data support continuing the recommendation to inoculate on the fifth year since previous soybean crop. Our assay suggested tentative inoculate recommendations primarily for fields either without a history of soybean planting, or over 5 years since the previous soybean crop. However, we did identify one field in Eastern and one field in Western North Dokata with non-detectable levels of rhizobia despite only 2 years since the previous soybean crop (** in Figure 7, Figure 8B). This indicates that our tool to quantify rhizobium populations could be useful as an insurance case for scenarios when the typically expected dynamics of rhizobium populations don't hold up for a given field, risking poor nodulation. Before implementation of the tool, data should be gathered for fields with high and low populations of rhizobia to assess the impact of inoculation on nodulation and yield. By connecting inoculant field trials with the results from

the NDSoy2.0 assay in FY24 we hope to near implementation of the assay as a valuable agronomic tool to monitor rhizobium populations in the soil, and as a tool for farmers to assess the need to inoculate in a coming year.

Sample	Location	Region	Years Since Soybeans	Average Rhizobia per Gram	Overall Rhizobia	Tentative Inoculate Recommendation
S212	Grand Forks County	Eastern ND	3	34,662	Low	Next Year
S213	Grand Forks County	Eastern ND	2	142,955	Medium	No
S214	Grand Forks County	Eastern ND	2	152,673	Medium	No
S215	Grand Forks County	Eastern ND	1	52,403	Low	Next Year
S216	Grand Forks County	Eastern ND	Never	2,287	Low	Next Year
S217	Richland County	Eastern ND	2	Not Detected	Not Detected	Yes**
S218	Richland County	Eastern ND	2	40,972	Low	Next Year
S219	Richland County	Eastern ND	2	168,115	Medium	No
S228	Carrington	Eastern ND	Never	8,555	Low	Next Year
S230	Carrington	Eastern ND	7	Not Detected	Not Detected	Yes
S201	Williston	Western ND	2	2,497,138	High	No
S202	Williston	Western ND	Never	122,403	Med	No
S203	Burke County	Western ND	Never	Not Detected	Not Detected	Yes
S204	Burke County	Western ND	3	29,230	Low	Next Year
S205	Burke County	Western ND	1	10,437	Low	Next Year
S206	Hettinger	Western ND	2	Not Detected	Not Detected	Yes**
S207	Hettinger	Western ND	Never	Not Detected	Not Detected	Yes
S208	Hettinger	Western ND	6	Not Detected	Not Detected	Yes
S209	Morton County	Western ND	1	2,267,194	High	No
S210	Morton County	Western ND	3	2,888,989	High	No
S211	Grant County	Western ND	Never	Not Detected	Not Detected	Yes
S220	Williston	Western ND	3	130,396	Medium	No
S222	Williston	Western ND	Never	Not Detected	Not Detected	Yes
S224	Hettinger	Western ND	1	4,551,348	High	No
S226	Hettinger	Western ND	8	Not Detected	Not Detected	Yes

Figure 7. Rhizobium populations in fields sampled in 2023 by NDSoy2.0 assay. Rhizobia populations >1,000,000 cells per gram of soil were considered as high, >100,000 cells per gram were considered as medium, detectable but <100,000 as low. Tentative inoculation recommendations are suggested for fields where rhizobia is not detected, and a suggestion to consider inoculating if planting soybeans in the following year are suggested for fields where rhizobia populations are low.

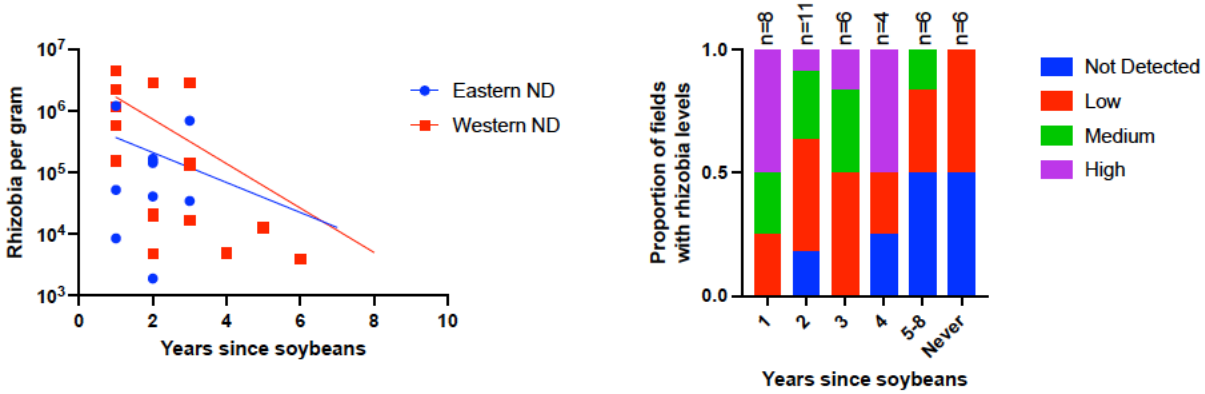

Figure 8. A (left), shows pooled data from 2022 and 2023 where detectable rhizobia were identified in fields compared to the number of years since soybeans were grown. A decline in populations is observed over time, with a significant drop at 4 years since the pervious soybean crop. B (right) shows the proportion of fields with high, medium, low or not detectable amounts of rhizobia based on the number of years since the previous soybean crop.