HER2 amplification Detection in Tumour Samples

HER2 Rx-plex

Product Line: INNOplex Rx

Cat. No.: AB-0117-01-Rx

25 tests per kit

Manufacturer: Applied Biotech Limited

Quality Control and Certification: Biotech INNOplextions Limited

Product Description

1. Intended Use

The HER2 Rx-plex kit is used to detect amplification of Human Epidermal Growth Factor Receptor 2 (HER2) in Formalin Fixed Paraffin Embedded (FFPE) human tissue samples and in RNA samples of human origin.

The kit can be used with Luminex LX-200 and Luminex MAGPIX system. The MAGPIX system is a clinical multiplex test system intended to measure and sort multiple signals generated in an in vitro diagnostic assay from a clinical sample. This instrument system is used with a specific assay to measure multiple analytes that aid in diagnosis.

FOR RESEARCH USE ONLY.

2. Principle of Technology

Investigations using RNA from archival formalin-fixed paraffin-embedded (FFPE) material is challenging due to the extent of degradation in these samples and also due to the processing variables during and following cut-up of the surgical samples. Formalin cross-links nucleic acids and proteins to preserve tissue integrity, resulting in poor quality of RNA. The branched-chain DNA (bDNA) assay QuantiGene® technology provides a platform to perform expression studies from minimal amount of archival FFPE material without amplification of target sequences, overcoming the limiting factor of expression studies using degraded RNA. This assay replaces enzymatic amplification of target RNA with hybridization of specific probes followed by amplification of the reporter signal, through DNA molecule scaffold formation on bound probe-target sequences. The capture and detection probes provide increased specificity during hybridization and the short recognition sequences are designed to capture short fragments of target RNA. In addition, the use of tissue homogenates directly into the assay, overcomes the low yield of RNA following RNA extraction and purification. The INNOPLEX Rx assay provides the possibility to multiplex the quantification of a panel of targets, generating datasets within one run. Analyses using mathematical algorithms, derive the relative gene expression values and interpret the resulting gene expression profile.

3. Product Description

Breast cancer classification is today supported by molecular markers categorising patients into four molecular classes, namely luminal A, luminal B, the human epidermal growth factor receptor 2 (HER2)-enriched and the basal types. Luminal A subtype is positive for oestrogen receptor (ER) and/or progesterone receptor (PgR) expression with low expression of Ki-67, while luminal B, apart from having an ER/PgR positive expression, includes also HER2 positive and negative subgroups associated with high Ki-67 expression. The HER2-enriched are well defined, with an exclusive high expression of HER2 receptor, due to the ERBB2 gene amplification, combined with low or absent ER and PgR. The basal type are in general negative for the latter 3 receptors, the triple negative breast cancer (TNBC) subtype.

Positivity to HER2 classifies the patients for trastuzamab treatment. A Multiplex Branched DNA assay has been optimised to quantify gene expression on degraded RNA derived from Formalin Fixed Paraffin Embedded (FFPE) breast cancer tissue [1]. The gene expression assay is a novel quick and multiplex method that can accurately classify the HER2 breast cancer subtype, omitting subjectivity of interpretation and minimising technical variation. This method has a wide range of possible applications in the diagnosis of tumours and is adapted to the current diagnostic workflow.

1. Grech G, Baldacchino S. Molecular Classification of Breast Cancer Patients Using Formalin-fixed Paraffin-embedded Derived RNA Samples. Journal of Molecular Biomarkers & Diagnosis. 2016;01(s8). doi:10.4172/2155-9929.s8-016.

4. Kit Contents

The components of the INNOPLEX Rx Assay Kit and storage conditions are listed in the Table below:

4.1 INNOPLEX Rx Sample processing part contains two parts: **Homogenizing Solution** (keep at room temperature, shipped in Box 3)and **Proteinase K** (keep at –20 °C and shipped in Box 1).

4.2 INNOPLEX Rx Reagent System containing the following:

Component	Description	Storage	Box
Pre-amplifier (PA1)	DNA in aqueous buffered solution	–20 °C	1
Amplifier (A1)	DNA in aqueous buffered solution	–20 °C	1
Blocking Reagent	Aqueous buffered solution with preservative	–20 °C	1
Capture probes	DNA bound to 96-well plate	–20 °C	1
Label probes	Oligonucleotide-alkaline phosphatase conjugate in aqueous buffer solution	–20 °C	1
Substrate *Lumigen® APS-5**	Chemiluminescent substrate	2-8 °C	2
Amplifier-Label Probe Diluent	Aqueous buffered solution with a protein containing preservative	2-8 °C	2
Lysis Mixture	Aqueous buffered solution with preservative	15-30 °C	3
Wash Buffer 1 (WB1)	Aqueous solution	15-30 °C	3
Wash Buffer 2 (WB2)	Aqueous buffered solution	15-30 °C	3

* Thermo Fisher Quantigene substrate

4.3 INNOPLEX Rx Luminex bead kit containing the following:

Component	Description	Storage	Box
Capture Beads	Luminex beads conjugated with specific probes.	2-8 °C	2

4.4 INNOPLEX Rx Detection kit containing the following:

Component	Description	Storage	Box
SAPE	Streptavidin, R-Phycoerythrin Conjugate	2-8 °C	2
SAPE diluent	Aqueous buffered solution	15-30 °C	3

Note: The kit includes Plate Seals (Adhesive foil and plastic seal) shipped in Box 3.

Components are shipped as follows: Box 1 using dry ice, box 2 using cold packs and the rest at room temperature (box 3).

5. Preparation and materials required

- Plan sample plate and print plate map. Include controls and blanks.
- Make sure to wipe bench with alcohol followed by RNaseZap, use filter tips and pipettes dedicated for RNA handling.
- Place Temperature probe in Vortemp into a well of the 96-well plate and switch on Vortemp at 54°C with 600rpm shaking.
- **Validate** temperature after 1 hour. If the temperature is off calibrate and re-check after 1hour.
- Oven/incubator at 37°C

Materials (not supplied)

- Magnetic Separation plate (96-well plate, sterile, flat bottom, with edge for attachment to magnetic plate and untreated (Recommended: 243656 Thermo-Fisher - Nunc)
- Filter Tips
- Autoclaved yellow tips
- Deionised water
- Sterile RNAse free water
- Luminex Sheath fluid
- Luminex Calibration and Validation kits
- Refrigerated containers
- 15mL RNAse free, sterile tubes

Equipment

- High precision shaking Incubator (Recommended: Vortemp)
- Calibration Kit
- Hand-Held Magnetic plate washer
- Multichannel pipette (1-100)
- RNA dedicated Pipette set
- Oven at 37°C
- Sonicator
- Vortex
- Luminex
- Thermomixer with Plate attachment

6. Sample Collection, Storage and transport

Materials

- Homogenizing solution (Supplied)
- Proteinase K (Supplied)
- RNA-dedicated pipettes
- Filter tips

Equipment

- Tissue corer
- Vortex
- Thermomixer

Sample Preparation

1. Using a tissue block corer, core the FFPE block at the selected site (using H&E).
2. Prepare a master mix of 150µL homogenizing solution and 2.5µL proteinase K (60:1 ratio).
3. Use 60µL to 300µL of homogenizing mix for 25-125mm^2 of tissue area which translates to 0.5 - 2.5mm^3 of tissue volume. Minimum homogenizing solution to be used for 1 reaction is 100µL
4. Add the respective volume of homogenizing solution mix to each tube
5. Vortex for 10s at max speed (3500rpm) and centrifuge for a few seconds.
6. Ensure that all dissected tissue fragments are immersed in solution using the pipette tip.
7. Incubate at 65°C for 12-18 hours with shaking at 600rpm for 30s at 30 minute intervals.

8. The following day, label a second set of tubes while centrifuging the lysates at 14,000rpm for 5 minutes at room temperature.
9. Carefully aspirate the middle phase of the lysate which is the clear liquid between the waxy top layer and the tissue fragments at the bottom and transfer to the second tube. Avoid taking up wax or tissue/membrane fragments as this may reduce quality of results.
10. Store clear lysate -80°C. These can withstand at least 10 freeze-thaw cycles with negligible changes to expression and can be stored for up to two years.

NOTE: Examine homogenate - It should be clear and viscous. If it is still not after re-vortexing and centrifugation try re-heating to 65°C again and repeating steps 7 and 8.

7. Procedure

Day 1 - Hybridisation

Approximate time required: 2 hours

Before Starting

- Pre-warm lysis Mixture at 37°C for 30 minutes and mix gently.
- If tissue homogenates have been frozen, remove from freezer and thaw at room Temperature and incubate at 37°C for 30 minutes. Vortex samples at max speed following incubation.
- Calibrate Vortemp and set at 54°C. Allow 2 hours for Vortemp to reach 54°C.
- Prepare reagents:
    a. **Probe set and Blocking Reagent** - Thaw and vortex briefly, centrifuge briefly and keep on ice.
    b. **Proteinase K** - Keep on ice
    c. **Capture Beads** - Take out of storage right before use and protect from light. **Sonicate and vortex for 3 minutes respectively before use** (to avoid and eliminate bead clumps which leads to low bead counts and prevents set up of capturing scaffold which can give aberrant results)

1. Dilute tissue homogenates with **Homogenizing Solution** if or as needed (25µL per well)
2. Prepare **working bead mix** using Template table. Keep working bead mix at room temperature and protected from light. Vortex well before use (Do NOT keep on ice).
3. Vortex **Working Bead Mix** for 10s to mix, then dispense into the **Magnetic separation plate** (25µL per well). Pipette using a single channel pipette if less than 48 well plates. For more than 48 wells use a multichannel pipette and change tips each time.

4. Add 25µL tissue homogenate to each respective well into **Working bead mix** and add 25µL **Homogenizing solution** to 3 wells as Blanks.
5. Seal the Hybridisation plate using a clear plastic **Pressure Seal** (Film Thermaseal RTS x100, Part No: 765246).
6. Place plate in the Vortemp Incubate for **18-22hours** at 54 ± 1 °C at 600rpm.

Day 2 - Washing, Amplification and Reading

Approximate time required: 5 to 6 hours (3.5hours incubation)

Preparation

- Confirm that the Vortemp is still at 54°C
- Warm Amplifier Diluent at 37°C for 30 minutes and mix well by inversion
- Bring Label Probe Diluent and SAPE Diluent to room Temperature.
- Take out the **Pre-Amplifier** to thaw (-20°)

1. Prepare **Wash Buffer** (Nuclease-free water is recommended although deionized or sterile and deionized water is also good for the wash buffer as theoretically the RNA is stabilized over the beads now and sterility and RNase free environment are not as important)
    a. Volume required = 100µL per well by 3 washes by 4 times +10% = a total of **1320µL per well**
    b. Wash buffer is prepared by mixing **deionized sterile water** with **Wash Buffer Component 1** and **component 2** in the ratio of 31.5 : 0.1: 1.667
    c. Hence to make up 32mL of Wash buffer for 24 wells use **31.5mL Water** with **0.1mL of Component 1** and **1.67mL of Component 2**

    IMP NOTE: Do NOT mix buffer components but pipette and mix directly in water.

2. Centrifuge the **Pre-Amplifier** briefly to collect at the bottom and mix by gentle flicking (**DO NOT VORTEX -** this would break the branching backbone for signal amplification)
3. Prepare the **Pre-Amplifier Working Reagent:** 0.003 : 1 of **Amplifier Diluent** and 50µL per well are required.
    a. Hence for 24 wells (50µL x 24wells = 1200µL; +10% = 1320µL):
    add 4.5µL of **Pre-Amplifier** to 1500µL **Amplifier Diluent** (to use reagent reservoir)
    add 4µL of **Pre-Amplifier** to 1330µL **Amplifier Diluent** (to pipette individually)
    b. Invert tube several times to mix or mix by pipetting (**DO NOT Vortex**)

    NOTE: Amplifier Diluent is viscous, pipette slowly and ensure entire volume transfer.

4. Remove plate from Vortemp. Set Temperature to 50°C ± 1°C and set calibration setting if required
5. Mount and lock the plate on the **Hand-held Magnetic Plate Washer** and wait 1 minute for the beads to settle at the bottom.

WASHING STEPS (Steps 6-8)

6. Empty the wells by inverting the mounted plate over the sink and blot gently facing down on a tissue paper to remove residual solution.
7. Add 100µL of **1x Wash Buffer** into each well and wait 15 seconds.
8. Repeat Steps 6 and 7 two times for a total of three washes and discard wash solution and blot well.

9. Add 50µL of **Pre-Amplifier Working Reagent** to each well with a single channel pipette or using a multi-channel pipette if a considerable amount of samples is being processed.
10. Seal the plate with a **Foil Plate Seal** (Sigma part no:- Z721549-100EA) and **shake** at 800rpm for 1 minute at room temperature.
11. Incubate for 1 hour in the Vortemp at 50°C ± 1°C with shaking at 600rpm.

12. Centrifuge the **Amplifier** briefly to collect at the bottom and mix by gentle flicking (**DO NOT VORTEX -** this would break the branching backbone for signal amplification)
13. Prepare the **Amplifier Working Reagent:** 0.003 : 1 of **Amplifier Diluent** and 50µL per well are required.
    a. Hence for 24 wells (50µL x 24wells = 1200µL; +10% = 1320µL):
       add 4.5µL of **Amplifier** to 1500µL **Amplifier Diluent** (to use reagent reservoir)
       add 4µL of **Amplifier** to 1330µL **Amplifier Diluent** (to pipette individually)
    b. Invert tube several times to mix or mix by pipetting (**DO NOT Vortex**)

    NOTE: Amplifier Diluent is viscous, pipette slowly and ensure entire volume transfer.

14. After the 1 hour incubation, remove the plate from the Vortemp, place on the magnetic plate washer and remove the seal. Repeat the Washing steps 6-8.
15. Add 50µL of **Amplifier Working Reagent** to each well with a single channel pipette or using a multi-channel pipette if a considerable amount of samples is being processed.
16. Seal the plate with a **Foil Plate Seal** (Sigma part no:- Z721549-100EA) and **shake** at 800rpm for 1 minute at room temperature.
17. Incubate for 1 hour in the Vortemp at 50°C ± 1°C with shaking at 600rpm.

18. Thaw and centrifuge the **Label Probe** briefly to collect at the bottom and vortex for 10 seconds.
19. Prepare the **Label Probe Reagent:** 0.003 : 1 of **Label Probe Diluent** and 50µL per well are required.
    a. Hence for 24 wells (50µL x 24wells = 1200µL; +10% = 1320µL):
       add 4.5µL of **Label Probe** to 1500µL **Label Probe Diluent** (to use reagent reservoir)

add 4µL of **Label Probe** to 1330µL **Label Probe Diluent** (to pipette individually)

b. Vortex for 10s at maximum speed 3000rpm

20. After the 1 hour incubation, remove the plate from the Vortemp, place on the magnetic plate washer and remove the seal. Repeat the Washing steps 6-8.
21. Add 50µL of **Label Probe** to each well with a single channel pipette or using a multi-channel pipette if a considerable amount of samples is being processed.
22. Seal the plate with a **Foil Plate Seal** (Sigma part no:- Z721549-100EA) and **shake** at 800rpm for 1 minute at room temperature.
23. Incubate for 1 hour in the Vortemp at 50°C ± 1°C with shaking at 600rpm.

24. Usually this is a good time to switch on the Luminex instrument and run Calibration and Validation to make sure it is in correct working order (follow Luminex Setting up procedure below and the Luminex User Instructions).

25. Vortex the **SAPE** for 10s at full speed to mix and centrifuge briefly.
26. Prepare the **SAPE Working Reagent:** 0.003 : 1 of **SAPE Diluent** and 50µL per well are required.
    a. Hence for 24 wells (50µL x 24wells = 1200µL; +10% = 1320µL):
       add 4.5µL of **SAPE** to 1500µL **SAPE Diluent** (to use reagent reservoir)
       add 4µL of **SAPE** to 1330µL **SAPE Diluent** (to pipette individually)
    b. Vortex for 15s at maximum speed 3000rpm and protect from light

    NOTE: SAPE should be bright pink in colour, if it turns brownish it may have gone bad.

27. After the 1 hour incubation, remove the plate from the Vortemp, place on the magnetic plate washer and remove the seal. Repeat the Washing steps 6-8.

28. Add 50µL of **SAPE Working Reagent** to each well with a single channel pipette or using a multi-channel pipette if a considerable amount of samples is being processed.
29. Seal the plate with a **Foil Plate Seal** and incubate at room temperature and 800rpm for 1 minute.
30. Incubate at ROOM Temperature for 30minutes with shaking at 600rpm.

31. Repeat Wash Steps 6-8 using the **SAPE Wash Buffer**.
32. Add 130µL of **SAPE Wash Buffer** to each well seal with a **Foil Plate Seal**. If plate is not going to be read immediately wrap also in foil to avoid quenching signals.

33. Shake plate at 800rpm at room temperature for 3 minutes just before loading into the Luminex instrument and reading.
34. The plate can be stored wrapped in foil in the fridge for a week without any loss in expression values. Top up with SAPE Wash Buffer and store in the fridge. When taking out, replace wash buffer after attaching the plate to the Magnetic Hand-Held Washer, shake for 800rpm for 3 minutes at room temperature and read.

Set Luminex with the following parameters using the INNOA Rx HER2 panel protocol for the bead designation:

Sample Size:	100µL
DD Gate:	5,000 - 25,000
Timeout:	60 seconds
Bead Event/ Bead Region:	100

Data Analysis

1. Observe bead counts. Omit low bead count samples.
2. The software will average out blank wells and work out SDs and LOD (blank average + (3xSD))
3. Values lower than LOD will be regarded as undetected.
4. The software will compare control readings with previous runs and analyse control regression and dilution-signal ratios.
5. Exported Raw Data is normalised to the geomean of housekeeping genes.
6. The software will provide the HER2 amplification status as an OUTPUT result.

NOTE: The software will detect saturation levels and low sample amount. The HiPMT detection can be used to increase detected signals hence sensitivity. Delta Cal temperature of Luminex LX200 should remain in the range of ± 2°C, otherwise errors are introduced while reading.

Luminex Setting up

Luminex Start-up

1. Prepare Luminex maintenance plate with Water and Ethanol and calibration and verification reagents
2. NOTE: Sonicate and vortex Calibration and verification reagents before for 3 minutes each.
3. Go to the **'Maintenance Page'** and select **Calibration and Verification** and drop (**4-5** drops) the calibrators as indicated on the provided calibration plate.
4. Insert the plate into the Luminex using the Eject/Retract button
5. Run a **Flush** and **Prime** and then the **Calibration and Verification** procedure. Follow the Instructions in the calibration and verification kits.

Luminex shut down

Replace the test plate with water ethanol water in large wells of the calibration plate and prime and run the washing procedure to clean tubing.

Turn off Luminex analyser and its respective PC.

Templates

Date: _____ Run Name: _____

Working Bead mix

Order	Reagent	1 well (µL)	x _____	
1	**Nuclease-free water**	4.25		
2	**Lysis mixture**	16.65		
3	**Blocking Reagent**	1		
4	**Proteinase K**	0.1		
5	**Capture Beads - Vortex before adding**	0.5		
6	**2.0 Probe Set**	2.5		
Total		25		

Table 1: Working Bead mix. Lysis mixture must always be 1/3 of total volume. Water volume can be replaced with tissue homogenate volume. Recommended mix has 5µL more water and 20µL of tissue Homogenate is added instead.

Plate Map

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

3 BLANKS, no bead control and positive control, negative control

Notes

- Blocking reagent contains BSA to block any non-specific binding sites.

8. Sensitivity and Specificity

To evaluate the inter-run variability of the INNOPLEX Rx assay, the test was run using RNA derived from cell lines with known receptor status. Inter-run regression analysis using the assay and other probes (n=22), including Estrogen receptor probe, provide evidence that the gene expression results using the customised probe sets perform well between runs with an r^2 >0.99 (**Figure 1A**). In addition, correlation (r^2) of normalised expression data using the INNOPLEX Rx assay and qPCR, on the previously quantified genes, was >0.86 (data not shown). Using serial sections of an unstained sample and the same coordinates from the H&E stained slide show a high correlation of gene expression using the 22-plex assay with an r^2 >0.98 (**Figure 1B**).

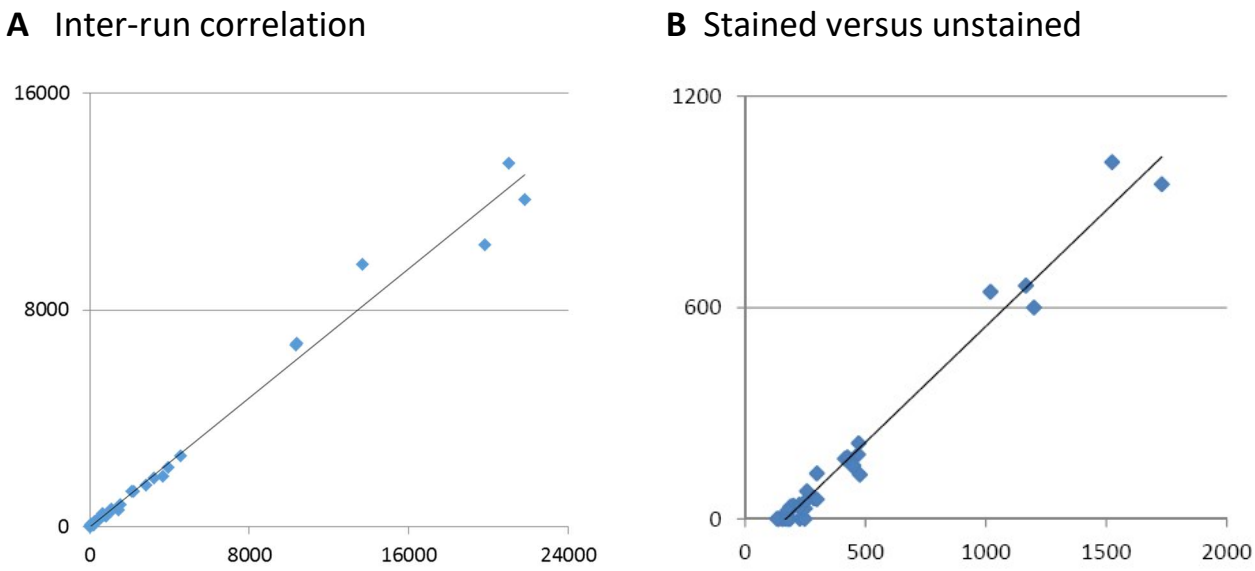

Figure 1: INNOPLEX Rx Gene expression (Mean Fluorescence Intensity) using cell line derived RNA and Formalin Fixed Paraffin Embedded (FFPE) derived RNA. **A.** Inter-run correlation of gene expression using purified RNA derived from a breast cancer cell line MDA-MB-453. **B.** Samples were microdissected and the same sample was analysed following H&E staining (y-axis) or unstained (x-axis). The relative expression levels did not differ and hence the assay can be used on stained microdissected FFPE sample lysates.

HER2 (ERBB2) expression correlates with that of other well established/benchmark techniques

The receptor status of tumours was used as an internal control of the expression runs. The results obtained using the INNOPLEX Rx Plex assay were correlated with known ERBB2 status using immunohistochemical (IHC) and fluorescence *in situ* hybridisation (FISH) results (defined as a signal ratio of ERBB2 probe to CEP17 control probe). The expression data correlates with both IHC (n=37) and FISH (**Figure 2**; n=11) results using Spearman's rho non-parametric correlation and Pearson's parameteric correlation, respectively. In addition, oestrogen receptor (ESR1) expression correlates strongly with IHC results (Spearman's rho: 0.878 p-value <0.000; data not shown).

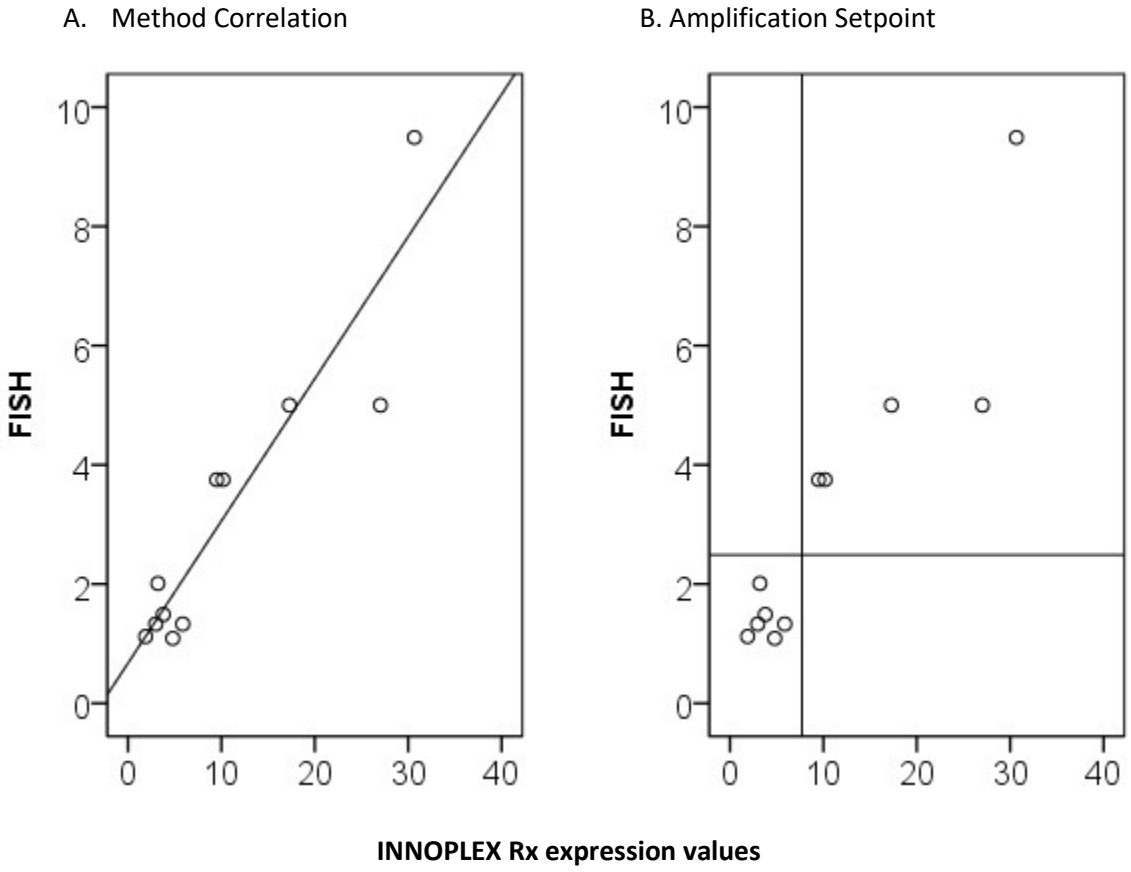

Figure 2: Comparison between normalized INNOPLEX Rx expression values (x-axis) and FISH ERBB2 results. ERBB2 expression of FFPE-derived RNA lysates using INNOPLEX Rx Plex assay correlates with FISH with an $r^2 = 0.861$ **[A].** Thresholds (lines) are set to diagnose ERBB2 (HER2) enriched cases based on FFPE-derived RNA samples **[B].**

This assay has an average sensitivity and specificity of 100% for the determination of HER2 status.

9. Contact Details

For further questions please contact our technical support at ceo@appliedbiotechltd.com

For sales enquires contact: business@appliedbiotechltd.com
For administrative and sales issues please contact: business@appliedbiotechltd.com