

Ion ReproSeq™ PGS Kits

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Note: For safety and biohazard guidelines, see the “Safety” appendix in the *Ion ReproSeq™ PGS Kits User Guide* (Pub. No. MAN0013762). Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

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Extract and amplify the gDNA

Extract genomic DNA

1. Prepare, by FACS or micro-manipulation, 1–10 cells per sample in up to 2.5 µL 1X PBS or Low TE, then pipet the cells into wells of a 96-well optical reaction plate.
2. Add Cell Extraction Buffer (green cap) to each sample well to bring the total volume to 5 µL.
3. (Optional) Prepare a Non-Template Control (NTC) by adding 2.5 µL 1X PBS to 2.5 µL Cell Extraction Buffer.
4. Prepare an Extraction Enzyme Master Mix in a 1.5-mL tube on ice according to the following table. Scale the volume according to the number of samples (N) and NTCs you have. Vortex briefly, then centrifuge to collect liquid at the bottom of the tube.

Component	Volume per reaction	Volume for N reactions ^[1]
Extraction Enzyme Dilution Buffer (violet cap)	4.8 µL	N × 4.8 µL × 1.1
Cell Extraction Enzyme (yellow cap)	0.2 µL	N × 0.2 µL × 1.1

^[1] 10% overage added to compensate for pipetting loss. Round to nearest 0.1 µL.

5. Add 5 µL Extraction Enzyme Master Mix to each cell or gDNA sample (10-µL final volume).
6. Seal the plate, then centrifuge at 1,000 × g for 30 seconds to collect liquid at the bottom of the wells.
7. Incubate the samples in a thermal cycler using the following temperature program:

Temperature	Time
75°C	10 minutes
95°C	4 minutes
22°C	Hold

8. Remove the plate, centrifuge at 1,000 × g for 30 seconds to collect liquid at the bottom of the wells, and place on ice or a cold block.

Proceed to "Pre-amplify the gDNA".

Pre-amplify the gDNA

1. Prepare a Pre-amplification Master Mix in a 1.5-mL tube on ice according to the following table.

Component	Volume per reaction	Volume for N reactions ^[1]
Pre-Amplification Buffer (red cap)	4.8 µL	N × 4.8 µL × 1.1
Pre-Amplification Enzyme (white cap)	0.2 µL	N × 0.2 µL × 1.1

^[1] 10% overage added to compensate for pipetting loss. Round to nearest 0.1 µL.

2. Carefully remove the film seal from the plate, then add 5-µL Pre-amplification Master Mix to each sample well (15-µL final volume).
3. Apply a new adhesive film, then centrifuge 1,000 × g for 30 seconds.

4. Cycle samples in a thermal cycler according to the following program:

Step	Temperature	Time ^[1]	Number of cycles
1	95°C	2 minutes	1
2	95°C	15 seconds	12
	15°C	50 seconds	
	25°C	40 seconds	
	35°C	30 seconds	
	65°C	40 seconds	
	75°C	40 seconds	
3	4°C	Hold	1

^[1] Cycling time is approximately 1 hour.

5. Remove the plate, centrifuge at $1,000 \times g$ for 30 seconds to collect liquid at the bottom of the wells, and place on ice or a cold block.

Proceed to "Amplify the libraries".

Amplify the libraries

1. If performing real-time PCR, prepare SYBR™ Green I/ROX dye mix:
 - a. Dilute the stock SYBR™ Green I reagent 1:1000 with Low TE buffer to make a 10X working solution.
 - b. Combine 10X SYBR™ Green I working solution and 25 μM ROX Reference Dye for the number of reactions (N) according to the following table:

Component	Volume per reaction	Volume for N reactions ^[1]
10X SYBR™ Green I working solution	0.5 μL	$N \times 0.5 \mu\text{L} \times 1.1$
25 μM ROX Reference Dye (or 2.5 μM, see below)	1.0 μL	$N \times 1.0 \mu\text{L} \times 1.1$
Nuclease-free Water (clear cap)	1.0 μL	$N \times 1.0 \mu\text{L} \times 1.1$

^[1] 10% overage added to compensate for pipetting loss. Round to nearest 0.1 μL.

IMPORTANT! Use ROX Reference Dye at 25 μM for the following real-time PCR instruments: Applied Biosystems™ 7300, 7900HT, StepOne™, StepOnePlus™, ABI PRISM™ 7000, and 7700 instruments.

Dilute ROX Reference Dye to 2.5 μM with Nuclease-free Water and use 1.0 μL per reaction for the following instruments: Applied Biosystems™ 7500 and ViiA™ 7 instruments, and Agilent™ Mx3000P™, Mx3005P™, and Mx4000™ instruments.

2. Prepare the Ion SingleSeq™ Barcode Set 1 plate:
 - a. Thaw the plate for 10 minutes at room temperature.
 - b. Centrifuge the plate briefly to collect contents at the bottom of the wells.
 - c. Wipe the foil seal with 70% ethanol, then allow it to dry.

3. Prepare an Amplification Master Mix in a 1.5-mL tube on ice according to the following table.

Component	Volume per reaction	Volume for N reactions ^[1]
Amplification Buffer (orange cap)	27 μL	$N \times 27 \mu\text{L} \times 1.1$
Amplification Enzyme (blue cap)	0.5 μL	$N \times 0.5 \mu\text{L} \times 1.1$
SYBR™ Green I/ROX dye mix ^[2]	2.5 μL	$N \times 2.5 \mu\text{L} \times 1.1$

^[1] 10% overage added to compensate for pipetting loss. Round to nearest 0.1 μL.

^[2] Replace with Nuclease-free Water if performing endpoint PCR.

4. Remove the film seal from the sample plate, then add 30 μL Amplification Master Mix to each well (45-μL final volume).
5. Pierce the foil above the desired well of the Barcode Plate with a pipette tip. With a new tip, withdraw 5 μL of the Ion SingleSeq™ Barcode Adapter, then add to the appropriate sample (50-μL final volume). Repeat for each sample. Each Barcode Adapter is single-use only.
6. Adjust a pipettor to 30 μL, then mix the samples by pipetting up and down, using a new tip for each sample.

Note: If you used a non-optical reaction plate or PCR tube strip for cell lysis and pre-amplification, you can transfer samples to an optical plate at this point to perform real-time PCR amplification.
7. Apply a new film seal to the plate and briefly centrifuge to collect liquid at the bottom of the wells.
8. Cycle the samples in the thermal cycler using the following program:

Step	Temperature	Time	Number of cycles
1	95°C	3 minutes	1
2	95°C	20 seconds	4
	50°C	25 seconds	
	72°C	40 seconds	
3	95°C	20 seconds	12 ^[1]
	72°C	55 seconds	
4	4°C	Hold	1

^[1] Acquire fluorescence data at this step if monitoring amplification in real-time.

9. Remove the plate, centrifuge at $1,000 \times g$ for 30 seconds to collect liquid at the bottom of the wells, and place on ice or in a cold block.

Proceed to "Library pooling, purification, and quantification".

STOPPING POINT Sample libraries can be stored at -30°C to -10°C before proceeding to the next step.

Library pooling, purification, and quantification

Note: We recommend that you perform library pooling, purification, and quantification in one session. Do not store library pools before quantification.

Pool the libraries

Pool the libraries according to the PCR method you used to amplify the libraries. Before pooling, vortex the amplification reactions to mix, then centrifuge briefly to collect contents at the bottom of the wells or tubes.

If you used	Action
Standard endpoint PCR amplification	Add 5 μL of each library to a new 0.2-mL tube to create an equi-volume pool. Vortex the tube to mix and pulse-centrifuge to collect contents at the bottom of the tube.
Real-time PCR amplification	<p>Calculate the median C_t value of the libraries you want to pool and adjust the volumes of libraries deviating from the median C_t following these guidelines:</p> <ul style="list-style-type: none"> Libraries that fall within 1 C_t of the median should be added at the normal volume (5 μL). Libraries that fall $>3 C_t$s later than the median should not be included in the pool. It is likely that the input material was not present in the tube or well or was absorbed to the tube side. Libraries that fall ≥ 1 but $\leq 3 C_t$s later than the median should be added at 2X the normal volume (10 μL). Do not adjust more than 25% of the libraries in a pool (excluding libraries $>3 C_t$s later than median). If more than 25% of libraries fall 1 or more C_ts away from the median, adjust libraries with the most extreme C_t deviations until the 25% threshold is reached.

Note: When pooling fewer than 8 libraries, the pool volume drops below 40 μL . Add Nuclease-free Water to bring the final volume to 40 μL before library pool purification.

Purify the library pool

Note: Purify the *entire volume* of the library pool. For a pool of fewer than 8 libraries, bring the volume to 40 μL with Nuclease-free Water.

- Heat the library pool in a thermal cycler using the following program:

Step	Temperature	Time	Number of cycles
1	70°C	2 minutes	1
2	22°C	Hold	1

- Pulse-centrifuge the tube to collect contents, then transfer the heated library pool to a new 1.5-mL Eppendorf LoBind™ Tube.
- Total the volumes of libraries and water added to the pool, then add an equal volume of AMPure™ XP Reagent beads.
- Vortex briefly, pulse-centrifuge the tube to collect contents, then incubate for 5 minutes at room temperature.

- Place the tube in the DynaMag™-2 magnet, then wait 5 minutes for beads to aggregate to the side of the tube.
- Aspirate the supernatant carefully, then discard.
- Wash beads with 150 μL of 70% ethanol while the tube is still on the magnet.
- Incubate for 30 seconds.
- Aspirate, then discard the wash solution.
- Repeat steps 7–9, thoroughly removing all ethanol after the second wash.
- Allow the beads to dry at room temperature for 3–4 minutes with the tube on the magnet.
- Remove the tube from the magnet, then resuspend beads in 200 μL of Low TE by pipetting up and down.
- Incubate the tube at room temperature for 1 minute.
- Place the tube in the DynaMag™-2 magnet, then wait 2–3 minutes for beads to aggregate to the side of the tube.
- Transfer 195 μL of the supernatant containing the purified library pool to a new 1.5-mL Eppendorf LoBind™ Tube and place on ice. Avoid carryover of beads.

Quantify the library pool

Quantify the Ion SingleSeq™ library pool with the Qubit™ dsDNA HS (High Sensitivity) Assay Kit. See the *Qubit™ dsDNA HS Assay Kits User Guide* (Pub. No. MAN0002326) for more information.

- Prepare a Qubit™ working solution by diluting the Qubit™ dsDNA HS Reagent (Component A) 1:200 in Qubit™ dsDNA HS Buffer (Component B) in a plastic tube.
- Prepare the standards:
 - Add 190 μL Qubit™ working solution to two labeled Qubit™ Assay Tubes used for standards.
 - Add 10 μL of each Qubit™ standard (Components C and D) to the appropriate tube.
 - Mix by vortexing 2–3 seconds. Do not create bubbles.
- Prepare the unknown:
 - Aliquot 198 μL Qubit™ working solution to labeled Qubit™ Assay Tubes used for samples.
 - Add 2 μL of the library pool to the appropriate sample tube.
 - Mix by vortexing 2–3 seconds. Do not create bubbles.
- Incubate the tubes in the dark at room temperature for 2 minutes.
- Measure standards first to generate a standard curve, then measure the library pool. For the Qubit™ 2.0 instrument:
 - For the library pool: on the Qubit™ instrument, press **Calculate Stock Conc.**
 - Turn roller wheel to 2 (μL s original sample added to reagent).

- c. Adjust units to ng/μL, then record this value.
6. Convert ng/μL to nM by multiplying the ng/μL values obtained in step 5 by 6.06 nmol/mg.
7. Dilute the library pool to 1 nM.

Prepare template-positive Ion PGM™ Template IA Ion Sphere™ Particles (ISPs)

Before you begin

1. Set a heat block to 40°C, with water added to wells.
2. Thaw the Ion PGM™ Template IA Primer Mix S (black cap), and keep it and the Ion PGM™ Template IA Start Solution (purple cap) on ice while setting up the reaction.
3. Thaw the Ion PGM™ Template IA ISPs (orange cap) and Ion PGM™ Template IA Rehydration Buffer (white cap) and keep on ice while setting up the reaction.

Prepare the final library pool dilution

1. Dilute the 1-nM library pool prepared in the previous chapter 1:100 with Nuclease-free Water to generate a 10-pM library pool.
2. Transfer 50 μL of the 10-pM library pool to a new 0.2-mL PCR tube.
3. Heat the tube in a thermal cycler using the following program:

Step	Temperature	Time	Number of cycles
1	70°C	2 minutes	1
2	4°C	Hold	1

4. Remove the tube from the thermal cycler and place on ice.

Perform the IA reaction

1. Prepare Templating Solution in a 2-mL Eppendorf LoBind™ Tube on ice (or a cold block):

Order of addition	Component	Volume per reaction
1	Ion PGM™ Template IA ISP Dilution Buffer (yellow cap)	122 μL
2	Ion PGM™ Template IA Primer Mix S ^[1] (black cap)	8 μL
3	Ion PGM™ Template IA ISPs ^[2] (orange cap)	21 μL
4	Library pool (10 pM) ^[3]	18 μL
—	Total	169 μL

^[1] Do not use Primer Mix L (blue cap).

^[2] Vortex 30 seconds at maximum speed to resuspend immediately before addition.

^[3] From step 4 of the previous section.

2. Vortex the tube containing the Templating Solution for 2 seconds at maximum setting to mix, pulse-centrifuge, then return the tube to ice.
3. Invert the Ion PGM™ Template IA Rehydration Buffer (white cap) 3 times to mix, then use 720 μL to rehydrate the Ion PGM™ Template IA Pellet. Vortex for 2 seconds at maximum setting, then pulse-centrifuge to collect contents at the bottom of the tube. Place the rehydrated pellet on ice or a cold block.
4. Transfer the rehydrated Ion PGM™ Template IA Pellet to Templating Solution on ice, vortex for 2 seconds at maximum setting, then pulse-centrifuge.
5. Invert the Ion PGM™ Template IA Start Solution (purple cap) 3 times to mix, then add 300 μL to the Template/IA Solution using the reverse pipetting technique.
6. Vortex the tube 10 times in 1-second pulses at the maximum vortexer setting. Invert the tube, then repeat the ten 1-second pulses.
7. Pulse-centrifuge the tube to collect contents, then immediately place the tube on ice.
8. Start the IA reaction by gently placing the tube in the 40°C heat block. Ensure that the tube is immersed in water.
9. Incubate the IA reaction for 25 minutes at 40°C.

Recover the template-positive ISPs

1. Stop the IA reaction by removing the tube from the heat block and adding 650 μL of Ion PGM™ Template IA Stop Solution.
2. Vortex the tube well to mix contents thoroughly, then centrifuge the tube at 7,500 × g for 3 minutes.
3. Aspirate, then discard the supernatant, being careful not to disturb the pellet. Leave ~100 μL in the tube.
4. Resuspend the pellet in 1 mL Ion PGM™ Template IA Recovery Solution.
 - a. Pipette up and down to resuspend the pellet.
 - b. Add an additional 700 μL Ion PGM™ Template IA Recovery Solution, then vortex thoroughly.
5. Incubate for 5 minutes with vortexing 5 seconds every minute.
6. Centrifuge for 3 minutes at 12,000 × g.
7. Immediately remove, then discard all of the supernatant without disturbing the ISP pellet. Remove any bubbles before removing the bulk of the liquid to avoid frothing in subsequent steps.
8. Add 100 μL of the Ion PGM™ Template IA Wash Solution to the ISP pellet.

- Resuspend the templated ISPs completely by vortexing for 4 seconds at maximum speed, then pipet the ISP suspension up and down 4 times. Proceed to "Enrich the template-positive ISPs".

STOPPING POINT Store templated ISPs in Ion PGM™ Template IA Wash Solution at 4°C for up to one week.

Enrich the template-positive ISPs

Prepare reagents then fill the 8-well strip

Prepare Melt-Off Solution

Prepare fresh Melt-Off Solution by combining the components in the following order:

Order	Component	Volume
1	Tween™ Solution	280 µL
2	1 M NaOH	40 µL
—	Total	320 µL

IMPORTANT! Prepare Melt-Off Solution as needed, but appropriately dispose of the solution after 1 day.

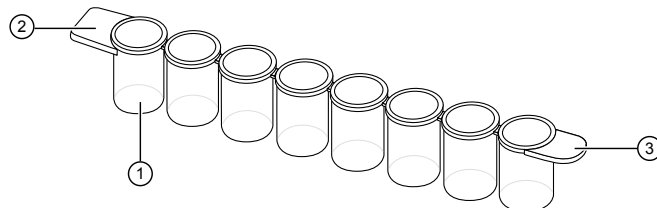
Wash and resuspend the Dynabeads™ MyOne™ Streptavidin C1 Beads

- Vortex the tube of Dynabeads™ MyOne™ Streptavidin C1 Beads for 30 seconds to resuspend the beads thoroughly, then centrifuge the tube for 2 seconds.
- Open the tube, then use a new tip to pipet the dark pellet of beads up and down until the pellet disperses. Immediately proceed to the next step.
- Transfer 13 µL of Dynabeads™ MyOne™ Streptavidin C1 Beads to a new 1.5-mL Eppendorf LoBind™ Tube.
- Place the tube on a magnet such as a DynaMag™-2 magnet for 2 minutes, then carefully remove and discard the supernatant without disturbing the pellet of Dynabeads™ MyOne™ Streptavidin C1 Beads.
- Add 130 µL of MyOne™ Beads Wash Solution to the Dynabeads™ MyOne™ Streptavidin C1 Beads.
- Remove the tube from the magnet, vortex the tube for 30 seconds, then centrifuge for 2 seconds.

Fill the 8-well strip

Note: If you stored template-positive ISPs at 2°C to 8°C, vortex the tube to resuspend the ISPs and pulse-centrifuge to collect contents. Pipet the solution up and down to resuspend the Ion PGM™ Template IA ISPs and transfer to Well 1 of the 8-well strip.

- Add the entire volume (~100 µL) of template-positive ISPs from the amplification reaction into Well 1 of the 8-well strip. Well 1 with the ISPs is on the left:



- Well 1
- Square-shaped tab
- Rounded tab

- If you have not done so already, assess the quality of the unenriched, template-positive ISPs using the Guava™ easyCyte™ 5 Flow Cytometer, or the Applied Biosystems™ Attune™ Acoustic Focusing Cytometer.
- Fill the remaining wells in the 8-well strip as follows:

Well number	Reagent to dispense in well
Well 1 (well nearest to the square-shaped tab)	Entire template-positive ISP sample (100 µL; prepared in step 1 of this procedure)
Well 2	130 µL of Dynabeads™ MyOne™ Streptavidin C1 Beads resuspended in MyOne™ Beads Wash Solution [prepared in "Wash and resuspend the Dynabeads™ MyOne™ Streptavidin C1 Beads"]
Well 3	300 µL of Ion PGM™ Template IA Wash Solution
Well 4	300 µL of Ion PGM™ Template IA Wash Solution
Well 5	300 µL of Ion PGM™ Template IA Wash Solution
Well 6	Empty
Well 7	300 µL of freshly-prepared Melt-Off Solution (prepared in "Prepare Melt-Off Solution")
Well 8	Empty

- Ensure that the square-shaped tab is on the left, then insert the filled 8-well strip with the 8-well strip pushed all the way to the right end of the slot of the Tray.

Prepare the Ion OneTouch™ ES

1. Load a new tip in the Tip Arm.
2. Ensure that the back/bottom end of the Tip Arm is not resting on top of the thumb screw, causing the Tip Arm to tilt forward.
3. Add 10 µL of Neutralization Solution to a new 0.2-mL PCR tube.
4. Insert the opened 0.2-mL PCR tube with the Neutralization Solution into the hole in the base of the Tip Loader.

Perform the Ion OneTouch™ ES run

Ensure that a new tip and opened 0.2-mL PCR tube with the Neutralization Solution have been loaded. Ensure that Well 1 (ISP sample) is the left-most well and that the 8-well strip is pushed to the far-right position in the slot.

1. Pipet the contents of Well 2 up and down to resuspend the beads before starting the run.
2. If needed, power on the Ion OneTouch™ ES, then wait for the instrument to initialize. The screen displays “rdy”.
3. Press **Start/Stop**.
4. At the end of the run, the instrument displays “End” and beeps every 60 seconds. Press the **Start/Stop** button to silence this alarm, then reset the Ion OneTouch™ ES for the next run.
5. **Immediately after the run**, securely close and remove the PCR tube containing the enriched ISPs.
6. Mix the contents of the PCR tube by gently inverting the tube five times.
7. Remove the used tip and the 8-well strip.

Sequence or store the template-positive ISPs

- Sequence using the Ion PGM™ Hi-Q™ Sequencing Kit, bundled in the Ion ReproSeq™ PGS Kit. Proceed to “Create a Planned Run”.

or

- Store the enriched ISPs at 2°C to 8°C for up to 3 days.

Create a Planned Run

1. Open the Torrent Browser on the Torrent Server connected to your sequencer.
2. Select the **Plan** tab, select **Templates**, then click **Whole Genome** from the list of applications on the left side of the page.
3. Select **Ion ReproSeq Aneuploidy** from the list of Planned Run templates.

4. The Ion ReproSeq™ Aneuploidy template will auto-populate settings in the Application, Kits, and Plugins tabs in the Planned Run wizard appropriately.

Note: Select the appropriate chip type you are using in the **Kits** tab. Chip type is set to Ion 318™ Chip v2 by default.

In the **Plan** tab,

- a. Enter a new **Run Plan Name**.
- b. Select **hg19(Homo sapiens)** from the **Reference Library** drop-down list.
- c. Select **None** from the **Target Regions** and **Hotspot Regions** dropdown lists.
- d. Enter the number of barcodes in your Sample Set.
- e. Select a Barcode and enter a unique Sample Name for each sample.
- f. Click **Plan Run** in the lower right corner to save the Planned Run.

Note: For details about creating a Planned Run, and enabling Ion Reporter™ auto-analysis, see the *Ion ReproSeq™ PGS Kits User Guide* (Pub. No. MAN0013762).

Clean, initialize, and run the Ion PGM™ Sequencer

See the *Ion PGM™ Hi-Q™ Sequencing Quick Reference* (Pub. No. MAN0010863) for a brief protocol for cleaning, initializing, and running the Ion PGM™ Sequencer, and for Ion PGM™ chip loading. Procedural details are also in the *Ion PGM™ Hi-Q™ Sequencing Kit User Guide* (Pub. No. MAN0009816). When 250 flows are set up in Planned Runs, 3 chips can be sequenced per initialization.

Launch an Ion Reporter™ analysis

See the *Ion ReproSeq™ PGS Kits User Guide* (Pub. No. MAN0013762) for detailed instructions for launching an Ion Reporter™ analysis using the ReproSeq Low-pass whole-genome aneuploidy workflow to analyze your samples.

If you are analyzing your samples manually, follow the instructions below. If you planned your run for automatic analysis with Ion Reporter™ Software, proceed to step 6.

1. Import your samples into Ion Reporter™ Software using the Ion Reporter™ Uploader plugin.
2. In the Ion Reporter™ **Home** tab, click **Launch analysis** after the Ion Reporter™ Uploader plugin has completed.
3. In the **Workflow** tab, select **Aneuploidy** from the **Applications** menu, and **Ion** from the **Workflow** menu to view the Ion aneuploidy workflows. Select **ReproSeq Low-pass whole-genome aneuploidy** from the list, then click **Next**.
4. In the **Samples** tab, select the sample(s) you want to run in your analysis from the list.
5. Click **Next** twice to advance through the **Plugins** tab to **Confirm & Launch**.

6. In the **Confirm & Launch** tab, enter a name for your analysis if you want to change the default name, then click **Launch Analysis**.
7. Review your results by selecting from the Analyses list after navigating to **Analysis ▶ Overview**. See the *Ion Reporter™ Software Help Guide* (Pub. No. MAN0013516) for further details.

Limited product warranty

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Revision history: Pub. No. MAN0014061

Revision	Date	Description
F.0	7 November 2016	<ul style="list-style-type: none"> • Recommended input volume of 10 pM library pool used in the IA reaction increased from 10 µL to 18 µL. • Topic organization in "Prepare template-positive Ion PGM™ Template IA Ion Sphere™ Particles (ISPs)" on page 4 restructured for ease of use.
E.0	6 April 2016	Ion Template IA 500 reagents and solutions cap colors updated.
D.0	21 December 2015	Changes in sequencing workflow made to align with user guide.
C.0	7 October 2015	Minor updates and rebranding.
B.0	9 August 2015	Configurations added for 5 additional kits for 314 and 316 chip configurations, with and without chip kits. Title changed correspondingly.
A.0	6 July 2015	New Quick Reference.

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